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(71) Applicant *(for all designated States except US)*: **THE JOHNS HOPKINS UNIVERSITY** [US/US]; 720 Rutland Avenue, Baltimore, MD 21205 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **WU, Tzyy-Choou** [—/US]; 11002 Nacirema Lane, Stevenson, MD 21153 (US). **HUNG, Chien-Fu** [—/US]; 6605 Sanzo Road, Baltimore, MD 21209 (US).

(74) Agent: **LIVNAT, Shmuel**; Venable, Baetjer, Howard & Civiletti, LLP, 1201 New York Avenue, NW, Suite 1000, P.O. Box 34385, Washington, DC 20043-9998 (US).

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(54) Title: NUCLEIC ACID DERIVED VACCINE THAT ENCODES AN ANTIGEN LINKED TO A POLYPEPTIDE THAT PROMOTES ANTIGEN PRESENTATION

(57) Abstract: Improved molecular vaccines comprise nucleic acid vectors that encode a fusion polypeptide that includes polypeptide or peptide physically linked to an antigen. The linked polypeptide is one that (a) promotes processing of the expressed fusion polypeptide via the MHC class I pathway and/or (b) promotes development or activity of antigen presenting cells, primarily dendritic cells. These vaccines employ one of several types of nucleic acid vectors, each with its own relative advantages: naked DNA plasmids, self-replicating RNA replicons and suicidal DNA-based on viral RNA replicons. Administration of such a vaccine results in enhance immune responses, primarily those mediated by CD8+ cytotoxic T lymphocytes, directed against the immunizing antigen part of the fusion polypeptide. Such vaccines are useful against tumor antigens, viral antigens and antigens of other pathogenic microorganisms and can be used in the prevention or treatment of diseases that include cancer and infections.

WO 2002/061113 A3

SUPERIOR MOLECULAR VACCINE BASED ON SELF-REPLICATING RNA, SUICIDAL DNA OR NAKED DNA VECTOR, THAT LINKS ANTIGEN WITH POLYPEPTIDE THAT PROMOTES ANTIGEN PRESENTATION

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

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BACKGROUND OF THE INVENTION

Field of the Invention

The present invention in the fields of molecular biology, immunology and medicine relates to chimeric nucleic acids encoding fusion proteins and their use as vaccines to enhance immune responses, primarily cytotoxic T lymphocyte (CTL) responses to specific antigens such as tumor antigens. The fusion proteins comprise an antigenic polypeptide fused to a protein that promotes processing via the MHC class I pathway and/or promotes development or activity of antigen presenting cells (APCs), primarily dendritic cells (DCs). Preparation of the foregoing nucleic acid constructs as naked DNA plasmids, self-replicating RNA replicons and suicidal DNA-based viral RNA replicons confer various advantages on these molecular vaccines.

Description of the Background Art

Antigen-specific cancer immunotherapy has emerged as a promising approach because it is capable of engendering specific immunity against neoplastic cells while sparing normal cells. Increasing evidence suggests that professional antigen-presenting cells (APCs), particularly dendritic cells (DCs), are central players in this process. An effective vaccine strategy includes targeting the tumor antigen to professional APCs that in turn activate antigen-specific T cells (for review, see (Chen, CH *et al., J Biomed Sci.* 5:231-52, 1998.).

Recently, DNA vaccines have become attractive as an approach for generating antigen-specific immunotherapy (for review, see (Robinson, HL *Vaccine* 15:785-778, 1997; Robinson, HL *et al., Semin Immunol. 9*:271-83, 1997; Pardoll, DM *et al., Immunity. 3*:165-9, 1995; Donnelly, JJ *et al., Annu Rev Immunol. 15:* 617-48, 1997). The advantages of naked DNA include purity, ease of preparation and stability. In addition, DNA-based vaccines can be prepared inexpensively and rapidly in large-scale. Furthermore, multiple DNA vaccines can be administered simultaneously. However, naked DNA vaccines raise concerns such as potential integration into the host genome and cell transformation. Because they do not have the intrinsic ability to amplify *in vivo* as do viral vaccines, DNA vaccines may be more limited in their potency.

The present inventors conceived that a directing a DNA vaccine encoding an antigen (in the form of a fusion protein) to cells which activate immune responses, such as DCs, would enhance the vaccine's potency. Others demonstrated that linking DNA encoding the cytokine GM-CSF gene to DNA encoding an HIV or hepatitis C antigen enhanced the potency of DNA vaccines (Lee, AH *et al., Vaccine 17*: 473-9, 1999; Lee, SW *et al., J Virol. 72*: 8430-6, 1998). The chigmeric GM-CSF/antigen is believed to act as an immunostimulatory signal to DCs, inducing their differentiation from an immature form (Banchereau, J *et al., Nature* 392: 245-52, 1998). Since DCs and their precursors express high levels of GM-CSF receptors, the chimeric GM-CSF/antigen should target and concentrate the linked antigen to the DCs and further improve the vaccine's potency.

Use of self-replicating RNA vaccines (RNA replicons) has also been identified as an important strategy in nucleic acid vaccine development. RNA replicon vaccines may be derived from alphavirus vectors, such as Sindbis virus (Hariharan, MJ *et al.,* 1998. *J Virol* 72:950-8.), Semliki Forest virus (Berglund, PM *et al.,* 1997. *AIDS Res Hum Retroviruses* 13:1487-95; Ying, HT *et al.,* 1999. *Nat Med* 5:823-7.) or Venezuelan equine encephalitis virus (Pushko, PM *et al.,* 1997. *Virology* 239:389-401). These self-replicating and self-limiting vaccines may be administered as either (1) RNA or (2) DNA which is then transcribed into RNA replicons in cells transfected *in vitro* or *in vivo (*Berglund, PC *et al.,* 1998. *Nat Biotechnol* 16:562-5; Leitner, WW *et al.,* 2000. *Cancer Res* 60:51-5).

Self-replicating RNA infects a diverse range of cell types and allows the expression of a linked antigen of interest at high levels (Huang, HV 1996. *Curr Opin Biotechnol* 7:531-5) Because viral replication is toxic to infected host cells, such self-replicating RNA preparations eventually causes lysis of the transfected cells (Frolov, I *et al.,* 1996. *J Virol* 70:1182-90). These vectors cannot integrate into the host genome, and therefore do not raise concerns of associated with naked DNA vaccines. This is particularly important for vaccine development where target proteins are potentially oncogenic, such as human papillomavirus (HPV) E6 and E7 proteins.

The present inventors and their colleagues recently demonstrated that linkage of HPV-16 E7 antigen to *Mtb* heat shock protein 70 (Hsp70) leads to the enhancement of DNA vaccine potency (Chen, CH *et al.,* 2000. *Cancer Research* 60:1035-1042). (See also co-pending patent applications USSN 09/501,097, filed 09 February 2000; and USSN 099/421,608, filed 20 October 1999, all of which are incorporated by reference in their entirety.) Immunization with HSP complexes isolated from tumor or virus-infected cells induced potent anti-tumor immunity (Janetzki, S *et al.,* 1998. *J Immunother 21*:269-76) or antiviral immunity (Heikema, AE *et al., Immunol Lett* 57:69-74). In addition, immunogenic HSP-peptide complexes dould be reconstituted *in vitro* by mixing the peptides with HSPs (Ciupitu, AM *et al.,* 1998. *J Exp Med* 187:685-91). Furthermore, HSP-based protein vaccines have been created by fusing antigens to HSPs (Suzue, K *et al.,* 1996. *J Immunol* 156:873-9). The results of these investigations

point to HSPs a attractive candidates for use in immunotherapy. However, prior to the present inventors' work, HSP vaccines were all peptide/protein-based vaccines or, in more recent cases, were in the form of naked DNA. To date, there have been no reports of HSPs incorporated into self-replicating RNA vaccines.

Another molecule that stimulates growth of DC precursors and can help in generating large numbers of DCs *in vivo* is Flt3-ligand ("FL") (Maraskovsky, E *et al., J Exp Med 184*: 1953-62, 1996, Shurin, MR *et al., Cell Lmmunol. 179:* 174-84, 1997). FL has emerged as an important molecule in the development of tumor vaccines that augment numbers and action of DCs *in vivo*. Flt3, a murine tyrosine kinase receptor, first described in 1991 (Rosnet, O *et al., Oncogene.* 6: 1641-50, 1991), was found to be a member of the type III receptor kinase family which includes -kit and c-fms (for review, see (Lyman, SD *Curr Opin Hematol.* 5:192-6, 1998). In hematopoietic tissues, the Flt3expression is restricted to the CD34+ progenitor population. Flt3 has been used to identify and subsequently clone the corresponding ligand, Flt3-ligand or "FL" (Lyman, SD *et al., Cell. 75:* 1157-67, 1993; Hannum, C *et al., Nature. 368:* 643-8, 1994).

The predominant form of FL is synthesized as a transmembrane protein from which the soluble form is believed to be generated by proteolytic cleavage. The soluble form of FL (the extracellular domain or "ECD") is functionally similar to intact FL (Lyman, SD *et al., Cell. 75*: 1157-67, 1993). These proteins function by binding to and activating unique tyrosine kinase receptors. Expression of the Flt3 receptor is primarily restricted, among hematopoietic cells, to the most primitive progenitor cells, including DC precursors. The soluble ECD of FL induced strong anti-tumor effects against several murine model tumors including fibrosarcoma (Lynch, DH *et al., Nat Med. 3*: 625-31, 1997), breast cancer (Chen, K et al *Cancer Res. 57*: 3511-6, 1997; Braun, SE *et al., Hum Gene Ther. 10:* 2141-51, 1999), liver cancer (Peron, JM *et al., J Immunol. 161*: 6164-70, 1998), lung cancer (Chakravarty, PK *et al., Cancer Res. 59*: 6028-32, 1999), melanoma and lymphoma (Esche, C *et al., Cancer Res. 58*: 380-3, 1998).

There is a need in the art for improved molecular vaccines, such as nucleic acid vaccines, that combine potency and safety. The present invention helps meet this need by its design of novel fusion or chimeric polypeptides and nucleic acids coding therefor, that link the antigen with specialized polypeptides that promote antigen presentation by various mechanisms and that exploit delivery of these constructs by various nucleic acid vectors.

Partial List of Abbreviations used

APC, antigen presenting cell; BHK, baby hamster kidney; CMV, cytomegalovirus; CTL, cytotoxic T lymphocyte; DC, dendritic cell; ECD, extracellular domain; ELISA, enzyme-linked immunosorbent assay; FL, Flt3 ligand; GFP, green fluorescent protein; HPV, human papillomavirus; HSP, heat shock

protein; Hsp70, mycobacterial heat shock protein 70; IFN-γ, interferon-γ; i.m., intramuscular(ly); i.v., intravenous(ly); MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; β-gal, β-galactosidase

SUMMARY OF THE INVENTION

Self-replicating RNA vaccines (RNA replicons) have emerged as an important strategy for nucleic acid vaccine development. The present inventors evaluated the effect of linking HPV type 16 (HPV-16) E7 as a model antigen to *Mycobacterium tuberculosis* (*Mtb*) heat shock protein 70 (Hsp70) on the potency of antigen-specific immunity generated by a Sindbis virus self-replicating RNA vector, SINrep5. The results indicated that this RNA replicon vaccine containing E7/Hsp70 fusion genes generated significantly greater E7-specific T cell-mediated immunity than vaccines comprising wild type E7 DNA.

HPV-16 E7 was selected as a model antigen for vaccine development because HPVs, particularly HPV-16, are associated with most cervical cancers. HPV oncogenic proteins, E6 and E7, are co-expressed in most HPV-containing cervical cancers and are important in the induction and maintenance of cell transformation. Therefore, vaccines targeting E6 or E7 provide an opportunity to prevent and treat HPV-associated cervical malignancies. HPV-16 E7 is a well-characterized cytoplasmic/nuclear protein that is more conserved than E6 in HPV-associated cancer cells; E7 has been tested in a variety of HPV vaccines.

Furthermore, *in vitro* studies demonstrated that E7 antigen from apoptotic cells that have been transfected with E7/Hsp70 RNA replicons is taken up by bone marrow-derived dendritic cells (DC's) and presented more efficiently through the MHC class I pathway compared to antigen from than apoptotic cells transfected by wild-type E7 RNA replicons.

Importantly, the fusion of Hsp70 to E7 converted a less effective vaccine into one with significant potency against E7-expressing tumors. This antitumor effect involved NK cells and $CD8^+$ T cells. Thus, fusion of a nucleic acid sequence encoding Hsp70 to nucleic acid encoding an antigen of interest in the form of a self-replicating RNA vaccine greatly enhances the potency of this vaccine.

Naked DNA vaccines represent an attractive approach for generating antigen-specific immunity because of their stability and simplicity of delivery. Concerns with DNA vaccines include potential integration into the host genome, cell transformation, and limited potency. The use of DNA-based alphaviral RNA replicons ("suicidal DNA vectors"), as disclosed herein, may alleviate concerns surrounding DNA integration or cell transformation since suicidal DNA vectors eventually cause lysis of the cells they transfect.

To further improve the potency of suicidal DNA vaccines, the present inventors linked Hsp70 to E7 (as a model antigen) using DNA-based Semliki Forest virus (SFV) RNA vector, pSCA1. This suicidal DNA vaccine containing E7/Hsp70 fusion DNA produced a significantly greater E7-specific T cell-

mediated immune response in mice than did vaccines containing the wild type E7 DNA alone. Importantly, this fusion converted a less effective vaccine into one with significant therapeutic potency against established E7-expressing metastatic tumors. The antitumor effect was dependent upon CD8+ T cells. Thus, linkage of Hsp70 to an antigen enhances the potency of a suicidal DNA vaccine.

Flt3 (fms-like tyrosine kinase 3)-ligand is an important cytokine in the development and differentiation of professional APCs, particularly DCs. A recombinant chimeric or fusion polypeptide molecule comprising the extracellular domain (ECD) of Flt3-ligand (FL) linked to an antigen targets the antigen to DCs and their precursors. Using HPV-16 E7 as a model antigen, the present inventors linked FL to E7 and caused stimulation of an antigen-specific immune response by a naked DNA vaccine administered intradermally via gene gun. Vaccines that included DNA encoding a chimeric FL-E7 fusion polypeptide dramatically increased the frequency of E7-specific $CD8^+$ T cells when compared to vaccines of only E7 DNA. Cells transfected *in vitro* with FL-E7 DNA presented E7 via the MHC class I pathway more efficiently than did cells transfected with wild-type E7 DNA. Furthermore, bone marrow-derived DCs pulsed with lysates of cells that had been transfected to express an FL-E7 fusion protein presented E7 (via the MHC class I pathway) more efficiently than did DCs pulsed with lysates of cells expresssing (after transfection) E7 protein alone. More importantly, this fusion construct rendered a less effective vaccine highly potent in inducing a therapeutic response against established E7-expressing metastatic tumors. The FL-E7 fusion vaccines mainly targeted $CD8^+$ T cells as anti-tumor effects were completely independent of CD4+ T cells. Thus, fusion of DNA encoding the ECD of FL to DNA encoding an antigen markedly enhances the potency of a DNA vaccine acting via CD8-dependent pathways.

In one embodiment, the antigen (*e.g.*, the MHC class I-binding peptide epitope) is derived from a pathogen, *e.g.*, it comprises a peptide expressed by a pathogen. The pathogen can be a virus, such as, *e.g.*, a papilloma virus, a herpesvirus, a retrovirus (*e.g.*, an immunodeficiency virus, such as HIV-1), an adenovirus, and the like. The papilloma virus can be a human papilloma virus; for example, the antigen (*e.g.*, the Class I-binding peptide) can be derived from an HPV-16 E7 polypeptide. In one embodiment, the HPV-16 E7 polypeptide is substantially non-oncogenic, *i.e.*, it does not bind retinoblastoma polypeptide (pRB) or binds pRB with such low affinity that the HPV-16 E7 polypeptide is effectively non-oncogenic when expressed or delivered *in vivo*.

In alternative embodiments, the pathogen is a bacteria, such as*Bordetella pertussis*; *Ehrlichia chaffeensis*; *Staphylococcus aureus*; *Toxoplasma gondii*; *Legionella pneumophila*; *Brucella suis*; *Salmonella enterica*; *Mycobacterium avium*; *Mycobacterium tuberculosis*; *Listeria monocytogenes*; *Chlamydia trachomatis*; *Chlamydia pneumoniae*; *Rickettsia rickettsii*; or, a fungi, such as, *e.g.*, *Paracoccidioides brasiliensis*; or other pathogen, *e.g.*, *Plasmodium falciparum*.

In another embodiment, the MHC class I-binding peptide epitope is derived from a tumor cell. The tumor cell-derived peptide epitope can comprise a tumor associated antigen, *e.g.*, a tumor specific antigen, such as, *e.g.*, a HER-2/neu antigen.

In one embodiment, the isolated or recombinant nucleic acid molecule is operatively linked to a promoter, such as, *e.g.*, a constitutive, an inducible or a tissue-specific promoter. The promoter can be expressed in any cell, including cells of the immune system, including, *e.g.*, antigen presenting cells (APCs), *e.g.*, in a constitutive, an inducible or a tissue-specific manner.

In alternative embodiments, the APCs are dendritic cells, keratinocytes, astrocytes, monocytes, macrophages, B lymphocytes, a microglial cell, or activated endothelial cells, and the like.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

All publications, patents, patent applications, GenBank sequences and ATCC deposits, cited herein are hereby expressly incorporated by reference for all purposes.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram showing the SINrep5 self-replicating RNA transcripts. A methylated M^7G, "cap" is located at the 5' end of the mRNA, followed by a sequence responsible for the self-replication (replicase), the gene of interest (i.e. E7, Hsp70, E7/GFP or E7/Hsp70), and a polyadenylated tail (AAAA).

Figure 2. Antigen ppecific $CD8^+$ T cell cytotoxic activity. Mice (5 per group) were immunized with various RNA vaccines via intramuscular (i.m.) injection. Splenocytes from each group (5 mice per group) were pooled 14 days after vaccination. To perform the cytotoxicity assay, pooled splenocytes from the various self-replicating RNA vaccines were cultured with E7 peptide (aa 49-57, RAHYNIVTF, SEQ ID NO:22, which includes a MHC class I epitope) for 6 days and used as effector cells. TC-1 tumor target cells were mixed with splenocytes at various effector/target (E/T) ratios. Cytolysis was determined by quantitative measurement of LDH release. The self-replicating RNA E7/Hsp70 vaccine generated significantly higher lysis than the other RNA vaccines ($p<0.001$). Error bars reflect 3 samples for each group. CTL assays shown are from one representative experiment of three performed.

Figure 3. IFN-γ secretion by E7-specific $CD8^+$ T cells. Mice were immunized i.m. with various self-replicating RNA vaccines. Splenocytes were collected 14 days after vaccination. Splenocytes from various self-replicating RNA vaccines were cultured *in vitro* with E7 peptide RAHYNIVTF, with or without peptide for 6 days. The culture supernatants were collected for measurement of IFN-γ concentration by ELISA. The $CD8^+$ T cells were induced by the MHC class I epitope of E7.

Splenocytes from the self-replicating E7/Hsp70 RNA group stimulated with the E7 RAHYNIVTF secreted the most IFN- γ compared to the other RNA vaccines ($p<0.001$, one-way ANOVA). Results from the ELISA are from one representative experiment of three performed.

Figure 4. IFN-γ secretion by E7-specific $CD4^+$ T cells. Splenocytes from mice vaccinated with various self-replicating RNA vaccines were cultured *in vitro* with E7 peptide containing the MHC class II epitope (aa 30-67, DSSEEEDEIDGPAGQAEPDRAHYNIVTFCCKCDSTLRL, (SEQ ID NO:23), or no peptide (control). The culture supernatants were collected for measurement of IFN-γ concentrations by ELISA. There was no significant increase in secretion of IFN-γ by splenocytes from the self-replicating E7/Hsp70 RNA group stimulated with the above peptide compared to the other RNA vaccines (one-way ANOVA). Results from the ELISA are from one representative experiment of three performed.

Figure 5. Various SINrep5 self-replicating RNA vaccines induce tumor protection. Mice (5 per group) were immunized i.m. with the RNA vaccines. Two weeks later, mice were challenged with TC-1 tumor cells i.v. (tail vein) at 10^4 cells/mouse. Mice were monitored twice weekly and sacrificed on day 21 after challenge. Lungs were dissected from the mice 35 days after vaccination with SINrep5, SINrep5-Hsp70, SINrep5-E7, SINrep5-E7 mixed with SINrep5-Hsp70, SINrep5-E7/GFP or SINrep5-E7/Hsp70 RNA. The mean number of tumor nodules on the lung surface of the vaccinated mice were counted, wher a decrease was an indication of vaccine effectiveness at controlling growth of a tumor expressin HPV-16 E7. There were fewer mean pulmonary nodules in mice vaccinated with self-replicating E7/Hsp70 RNA vaccines (0.1 μg, 1μg, and 10 μg) compred to mice vaccinated with the other RNA vaccines (10 μg) ($p<0.001$, one-way ANOVA). Self-replicating SINrep5-E7/Hsp70 RNA vaccines protect mice from intravenous tumor challenge even at the low dose of 0.1 μg whereas mice vaccinated with 10 μg of all the other vaccines developed numerous lung nodules. These tumor protection experiments were repeated three times with similar results.

Figure 6 shows representative photographs of lung tumors in each group. C57BL/6 mice were vaccinated i.m. with various RNA replicon-based vaccines (10 μg/mouse) and challenged with TC-1 tumor i.v., as above. Mice were sacrificed 35 days after vaccination. There are multiple grossly visible lung tumors in unvaccinated control mice and mice vaccinated with SINrep5 or SINrep5-E7 RNA vaccines. Lung tumors in SINrep5-E7/Hsp70 RNA vaccinated group are not evident at the magnification used.

Figure 7 shows the effect of lymphocyte subset depletion on the potency of self-replicating SINrep5-E7/Hsp70 RNA vaccine. Mice were immunized with 1 μg of thils vaccine i.m.. Two weeks after vaccination, mice were challenged with $1x10^4$ TC-1 cells/ mouse i.v. Depletions were initiated one week prior to tumor challenge and were carried out for 28 days. Three weeks after tumor challenge,

mice were sacrificed. The mean number of pulmonary nodules in vaccinated mice was determined as above. Depletion of $CD8^+$ T cells or of NK1.1 cells resulted in a higher number of pulmonary nodules vs. animals receiving control IgG_{2a} isotype antibody. The mean number of lung nodules in mice depleted of $CD4^+$ T cells were similar to those in mice receiving control antibody, indicating that $CD4^+$ T cells were not critical for the antitumor effect. Depletion of NK1.1+ cells had a greater impact on antitumor activity than did loss of $CD8^+$ T cells.

Figure 8 shows flow cytometric analysis of NK cells in mice immunized with various self-replicating SINrep5 RNA vaccines. Splenocytes were stained for CD3 and NK1.1 immediately, without stimulation. Fig. 8A shows numbers of NK cells in mice immunized with various self-replicating RNA vaccines. The percentage of NK cells among the spleen cells is indicated in the upper left corner. Fig. 8B is a histogram demonstratinge the percentages of NK cells in vaccinated mice. The percentage of NK cells in mice immunized with self-replicating RNA vaccines was higher than in unimmunized controls. There was no significant difference between the percentage of NK cells among mice given various self-replicating RNA vaccines. These results are from one representative experiment of two performed.

Figure 9 shows apoptotic death of host cells induced by self-replicating RNA vaccines. To determine if self-replicating RNA vaccines killed host cells, RNA transcribed *in vitro* from various SINrep5 plasmids was transfected into BHK21 cells. BHK 21 cells that were electroporated in the absence of RNA or unhandled BHK21 cells served as controls. The percentages of apoptotic and necrotic BHK21 cells were determined by staining with annexin V-FITC and propidium iodide (PI) and flow cytometric analysis. Transfection with SINrep5 RNA vaccines caused a decline in the percentages of apoptotic cells 24 hr to 72 hr after electroporation (representative with SIN5-E7/Hsp70 70.3±3.6% for 24 hr, 49.3±4.2% for 48 hr, 18.0±3.1% for 72 hr, $p<0.001$, one-way ANOVA). Thus no statistically significant difference were observed whe comparing the percentage of apoptotic cells transfected with various SINrep5 RNA vaccines. This experiment was repeated twice with similar results.

Figure 10. Enhanced MHC class I presentation of E7 to in bone marrow derived DCs pulsed with BHK21 cells transfected by SINrep5-E7/Hsp70 RNA as measured in CTL assays. BHK21 cells were electroporated with various self-replicating RNA constructs and co-cultured with BM-derived DCs. The DCs were then used as target cells for E7-specific $CD8^+$ T effector cells (28). Cytolysis was determined by quantitative measurements of LDH as described herein. Self-replicating E7/Hsp70 RNA vaccines generated significantly higher lysis (at 3:1 and 9:1 E/T ratios) compared to the other RNA vaccines ($p<0.001$). CTL assays shown here are from one representative experiment of two performed.

Figure 11 is a schematic diagram of pSCA1-E7, pSCA1-Hsp70 and pSCA1-E7/Hsp70 constructs. The DNA-based SFV replicon vector, pSCA1, encodes the alphaviral replicon from Semliki

Forest virus. E7, Hsp70, and E7/Hsp70 DNA were cloned into the BamHI/SmaI sites of pSCA1. The HCMV IE promoter with subgenomic promoter are indicated with arrows.

Figures 12A and 12B show measurement of E7-specific $CD8^+$ T cell precursors ("Tp") by intracellular cytokine staining and flow cytometric analysis. C57BL/6 mice were immunized with DNA-based self-replicating pSCA1-E7, pSCA1-Hsp70, pSCA1-E7/Hsp70, or pSCA1 without insert, using a gene gun and were boosted with the same regimen one week later. Fig. 12A): splenocytes from vaccinated mice were cultured with E7 peptide RAHYNIVTF (see above) overnight, stained for both CD8 and intracellular IFN-γ, and analyzed by flow cytometry. Mice vaccinated with E7/Hsp70 DNA generated the highest number of IFNγ-secreting $CD8^+$ "double positive" T cells compared to other groups. (Fig. 12B). Flow cytometry was performed in the presence (solid columns) and absence (open columns) of RAHYNIVTF peptide. Results are expressed as the mean number of IFN-γ-secreting $CD8^+$ T cells/$3x10^5$ splenocytes (± SEM). Results shown here are from one representative experiment of two performed.

Figures 13A and 13B show flow cytometry analysis of IFN-γ secreting or inteleukin-4 (IL-4)-secreting E7-specific $CD4^+$ T cells in mice vaccinated with various suicidal DNA vaccines. Mice were immunized as described in the description of Figure 12 (Fig. 13). Splenocytes from vaccinated mice were stimulated *in vitro* with the T-helper E7 peptide DSSEEEDEIDGPAGQAEPDRAHYNIVTFCCKCDSTLRL (see above) overnight and were stained for both CD4 and intracellular IFN-γ and subjected to flow cytometry. No significant differences in the frequency of E7-specific IFN-γ-secreting $CD4^+$ cells was observed in mice immunized with various recombinant DNA vaccines vs control. Fig. 13B: Splenocytes from vaccinated mice were cultured *in vitro* with the above E7 peptide overnight and stained for both CD4 and intracellular IL-4. The number of IL-4 secreting $CD4^+$ T cells was analyzed by flow cytometry. No significant difference in the frequency of IL-4 secreting E7-specific $CD4^+$ cells was observed among mice immunized with various recombinant DNA vaccines. This figure represents the mean value of three experiments ± SEM.

Figure 14 shows E7-specific antibody responses in mice immunized with various pSCA1 suicide DNA vaccines. E7-specific antibodies were measured with ELISA using a serial dilution of serum. The results from the 1:100 dilution are presented, showing mean absorbance (OD_{450}) (± SEM). The results are from one representative experiment of two performed.

Figure 15 shows *in vivo* tumor protection against the growth of TC-1 tumors. Mice were immunized with various suicidal DNA vaccines and inoculated with tumors as described below. 80% of mice vaccinated with the pSCA1-E7/Hsp70 suicidal DNA vaccine remained tumor-free 70 days after TC-1 challenge. The results shown here are from one representative experiment of two performed.

Figure 16A and 16B show results of *in vivo* tumor therapy against pre-existing metastatic TC-1 tumor cells. Mice were first inoculated i.v. with tumors and then treated with various suicidal DNA vaccines. Fig. 16A shows that the pSCA1-E7/Hsp70 group had the lowest number of pulmonary metastatic nodules of all the groups (ANOVA, $P<0.001$). Results are from one representative experiment of two performed. Fig. 16B shows representive lung tumors in each vaccinated group. Multiple grossly visible lung tumors were observed in unvaccinated control mice and mice vaccinated with Hsp70, E7, or vector DNA alone. No lung tumors were observed at this maginification in the pSCA1-E7/Hsp70 vaccinated group.

Figure 17. *In vivo* antibody depletion experiments determined the requirement for cells of certain lymphocyte subsets on the potency of the pSCA1-E7/Hsp70 suicidal DNA vaccine. Mice were inoculated i.v. with tumors and treated with the pSCA1-E7/Hsp70 suicidal DNA vaccine as described herein. CD4, CD8 or NK1.1 depletion were initiated one week prior to tumor challenge and lasted 40 days after tumor challenge. All naïve mice and mice depleted of $CD8^+$ T cells grew tumors within 10 days

Figure 18A and 18B show aschematic domain structure of the Flt3-ligand protein and FL-E7 fusion peptide (Fig. 18A) and the sequence of the FL-E7 construct, comprising the ECD of FL (Fig. 18B; SEQ ID NO:11 and 12). Residues 1-189 are FL-derived, residues 191-287 are E7-derived. The remaining residues (*e.g.*,, 288-302) are from the vector DNA.

Figure 19A and 19B. Intracellular cytokine staining with flow cytometry analysis to determine E7-specific $CD8^+$ T cell precursors in C57BL/6 mice . Mice were immunized with FL DNA, E7 DNA (E7), FL-E7 DNA or FL mixed with E7 DNA (FL+E7) via gene gun, or received no vaccination. For vaccinated mice, 2 μg DNA /mouse was administered twice. Splenocytes were harvested 7 days after the last DNA vaccination. E7-specific $CD8^+$ T cells. (Fig 19A) Splenocytes from vaccinated mice were cultured *in vitro* with E7 peptide (aa 49-57) overnight and were stained for both CD8 and intracellular IFN-γ. The number of IFN-γ secreting $CD8^+$ T cell precursors in mice immunized with various recombinant DNA vaccines was analyzed by flow cytometry. Mice vaccinated with FL-E7 DNA generated the highest IFN-γ^+ $CD8^+$ double positive T cells compared to other groups. (Fig. 19B) The number of IFN-γ-producing E7-specific $CD8^+$ T cells was determined using flow cytometry in the presence (solid columns) and absence (open columns) of E7 peptide (aa 49-57). Data are expressed as mean number of $CD8^+$ IFN-γ^+ cells/$3x10^5$ splenocytes ± SEM. The data from intracellular cytokine staining shown here are from one representative experiment of two performed.

Figure 20A and 20B. Flow cytometry analysis of IFN-γ secreting and IL-4-secreting E7-specific $CD4^+$ cells in mice vaccinated with various recombinant DNA vaccines. Mice were immunized as

described in Figure Legend 2. (Fig. 20A) Splenocytes from vaccinated mice were cultured *in vitro* with E7 peptide (aa 30-67) overnight and were stained for both CD4 and intracellular IFN-γ. The number of IFN-γ-secreting $CD4^+$ T cells was analyzed using flow cytometry. No significant difference in the frequency of E7-specific IFN-γ -secreting $CD4^+$ cells was observed in mice immunized with various recombinant DNA vaccines. (Fig. 20B) Splenocytes from vaccinated mice were cultured *in vitro* with E7 peptide (aa 30-67) overnight and stained for both CD4 and intracellular IL-4. The percentage of IL-4 secreting $CD4^+$ T cells was analyzed by flow cytometry. The IL-4 secreting activated mouse splenocytes (MiCK-2) from PharMingen were used as positive controls to assure the success of intracytoplasmic IL-4 staining for this study. The specificity of IL-4 staining was demonstrated by the absence of $CD4^+$ $IL\text{-}4^+$ T cells when the IL-4 antibody was omitted. No significant difference in the frequency of IL-4 secreting E7-specific $CD4^+$ cells was observed in mice immunized with various recombinant DNA vaccines. The intracellular cytokine staining shown here are from one representative experiment of two performed.

Figure 21. *In vivo* tumor protection experiments against the growth of TC-1 tumors. Mice were immunized with FL DNA , E7 DNA , FL-E7 DNA or FL mixed with E7 DNA (FL+E7) via gene gun and boosted with the same regimen one week later. One week after the last vaccination, mice were challenged with $1x10^4$ TC-1 cells/ mouse subcutaneously. Mice were monitored for evidence of tumor growth by palpation and inspection twice a week. 100% of mice receiving FL-E7 DNA vaccination remained tumor-free 60 days after TC-1 challenge. The data collected from the *in vivo* tumor protection experiments shown here are from one representative experiment of two performed.

Figure 22A and 22B. *In vivo* tumor treatment experiments against pre-existing metastatic TC-1 tumor cells. The mice were intravenously challenged with $1x10^4$ cells/mouse TC-1 tumor cells in the tail vein on day 0. Three days after challenge with TC-1 tumor cells, mice received 2 μg of FL DNA, E7 DNA, FL-E7 DNA, FL mixed with E7 (FL+E7), via gene gun or unvaccinated. One week later, these mice were boosted with the same regimen as the first vaccination. The mice were sacrificed on day 25. The FL-E7 group has (Fig. 22A) the least number of pulmonary metastatic nodules and (Fig. 22B) the lowest lung weight as compared with the other vaccinated groups (one-way ANOVA, $P<0.001$). The data obtained from these *in vivo* treatment experiments are from one representative experiment of two performed.

Figure 23. Representative gross pictures of the lung tumors in each vaccinated group. Following *in vivo* tumor treatment experiments against pre-existing metastatic TC-1 tumor cells, there are multiple grossly visible lung tumors in unvaccinated control mice and mice vaccinated with FL, wild-type E7 DNA or FL mixed with E7 DNA. The lung tumors in FL-E7 vaccinated group cannot be seen at the magnification provided in this figure.

Figure 24. *In vivo* antibody depletion experiments to determine the effect of lymphocyte subset on the potency of FL-E7 DNA vaccine. Mice were immunized with 2 μg FL-E7 DNA via gene gun and boosted with 2 μg FL-E7 DNA one week later. One week after the last vaccination, mice were challenged with $1x10^4$ TC-1 cells/ mouse subcutaneously. CD4, CD8 and NK1.1 depletions were initiated one week prior to tumor challenge and lasted 40 days after tumor challenge. Note: all of the unvaccinated mice and all of the mice depleted of $CD8^+$ T cells grew tumors within 14 days after tumor challenge. The data of antibody depletion experiments shown here are from one representative experiment of two performed.

Figure 25. CTL assays to demonstrate enhanced presentation of E7 through the MHC class I pathway in cells transfected with FL-E7 DNA. 293 D^bK^b cells were transfected with various DNA vaccines with lipofectamine and collected 40-44 hr after transfection. Transfected 293 D^bK^b cells were used as target cells while D^b-restricted E7-specific $CD8^+$ T cells were used as effector cells. CTL assays with various E/T ratios were performed. Note: The 293 D^bKb cells transfected with FL-E7 DNA generated significantly higher percentages of specific lysis as compared to 293 D^bKb cells transfected with other DNA vaccines. CTL assays shown here are from one representative experiment of two performed.

Figure 26. CTL assays to demonstrate enhanced MHC class I presentation of E7 in bone marrow derived dendritic cells pulsed with cell lysates containing chimeric FL-E7 protein. Bone marrow-derived DCs were pulsed with cell lysates from 293 D^bKb cells transfected with various DNA vaccines in different concentration (50 μg/ml, 10 μg/ml, 2 μg/ml, and 0.4 μg/ml) for 16-24 hrs. D^b-restricted E7-specific $CD8^+$ T cells were used as effector cells. CTL assays was performed at fixed E/T (9/1) ratio with $9x10^4$ of E7-specific T cells mixed with $1x10^4$ of prepared DCs in a final volume of 200 μl. Results of CTL assays were assessed using by quantitative measurements of LDH as described in the Materials and Methods. Note: DCs pulsed with lysates from cells transfected with FL-E7 DNA generated significantly higher percentages of specific lysis compared to DCs pulsed with lysates from cells transfected with other DNA vaccines. CTL assays shown here are from one representative experiment of two performed.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention provides compositions and methods for enhancing the immune responses, particularly cytotoxic T cell immune responses, induced by *ex vivo* or *in vivo* administration of chimeric polypeptides or, preferably, nucleic acid vaccines that encode these chimeric polypeptides. The preferred chimeric or fusion polypeptide comprises(1) at least one first polypeptide or peptide that, upon

introduction to cells of the host immune system, *in vitro* or *in vivo*, promotes (a) processing via the MHC class I pathway and/or (b) development or activity of APCs, primarily DCs, and (2) at least one second polypeptide or peptide that is an antigenic polypeptide or peptide in the host. As noted, in a preferred embodiment, the chimeric or fusion polypeptides are "indirectly" administered by administration of a nucleic acid that encodes the chimeric molecule; the nucleic acid construct, and thus the fusion protein, is expressed *in vivo*. The chimeric nucleic acids are administered in the form of DNA vaccines, either naked DNA or suicidal DNA, or a self-replicating RNA replicons.

The fusion protein comprises at least two domains or repeats thereof. The first domain again comprises a polypeptide that promotes (a) processing via the MHC class I pathway and/or (b) development or activity of APCs, and the second domain comprises a peptide or polypeptide, that includes one or several epitopes, derived from an antigen against which it is desired to induce an immune response.

For convenience, a polypeptide or peptide that promotes processing via the MHC class I pathway is abbreviated herein as "MHC-I-PP." A polypeptide or peptide that promotes development or activity of APCs, preferably DC's, is abbreviated DC-PP.

The exemplary MHC-I-PP protein describec herein is Hsp70. However, it is understood that any protein, or functional fragment or variant thereof, that has this activity can be used in the invention. A preferred fragment is a C-terminal domain ("CD") of Hsp70, which is designated "$Hsp70_{CD}$". One $Hsp70_{CD}$ spans from about residue 312 to the C terminus of Hsp70 (SEQ ID NO:4). A preferred shorter polypeptide spans from about residue 517 to the C-terminus of SEQ ID NO:4. Shorter peptides from that sequence that have the ability to promote protein processing via the MHC-1 class I pathway are also included, and may be defined by routine experimentation.

The second type of domain of the chimeric molecule comprises an antigenic peptide, which can be derived from a pathogen, a cancer cell, or any source to which induction, enhancement or suppression of an immune response is desired. In a preferred embodiment, the peptide comprises at least one MHC class I-binding peptide epitope that helps stimulate CD8+ CTLs and is recognized by such cells and their precursors.

The order in which the two (or more) component polypeptides of the fusion protein are arranged, and therefore, the order of the encoding nucleic acid fragments in the nucleic acid vector, can be altered without affecting immunogenicity of the fusion polypeptides proteins and the utility of the composition. For example, the Hsp70-encoding (or FL -encoding) DNA sequences may be located 5' or 3' to the target antigen-encoding sequences. In one embodiment, these polypeptide-encoding nucleic acid domains are in-frame so that the DNA construct encodes a recombinant fusion polypeptide in which the antigen is located N- terminal to the Hsp70 or FL derived polypeptide.

The vaccines of the present invention include, the antigenic epitope itself and an MHC-I-PP such as Hsp70 or its active domain (CD), or DC-PP intercellular spreading protein such as FL. In addition to the specific antigens and vectors employed in the Examples, the present invention is intended to encompass a vector such as naked DNA, naked RNA, self replicating RNA replicons and viruses including vaccinia, adenoviruses, adeno-associated virus (AAV), lentiviruses and RNA alphaviruses.

In addition to the MHC-I-PP and/or DC-PP, the vaccine construct of the present invention optionally, may also include

(a) an additional antigen targeting or processing signal such as proteins that promote intercellular transport, *e.g.*, VP22 protein from herpes simplex virus and related herpes viruses; an endoplasmic reticulum chaperone polypeptide such as calreticulin, ER60, GRP94 or gp96, well-characterized ER chaperone polypeptide that representatives of the HSP90 family of stress-induced proteins (Argon (1999) *Semin. Cell Dev..Biol.* 10:495-505; Sastry (1999) *J.* Biol. Chem. *274*:12023-12035; Nicchitta (1998) *Curr. Opin. Immunol.* 10:103-109; U.S. Patent 5,981,706)); cytoplasmic translocation polypeptide domains of pathogen toxins, such as domain II of *Pseudomonas* exotoxin ETA (ETAdII) or of similar toxins from *Diptheria, Clostridium, Botulinum, Bacillus, Yersinia, Vibrio cholerae*, or *Bordetella pertussis*; or active fragments or domains of any of the foregoing polypeptides.

(b) an immunostimulatory cytokine, preferably those that target APCs, preferably DC's, such as granulocyte macrophage colony stimulating factor (GM-CSF), or active fragments or domains thereof; and

(c) a costimulatory signal, such as a B7 family protein, including B7-DC (see commonly assigned U.S. patent application Serial No. 09/794,210), B7.1, B7.2, soluble CD40, *etc.*).

(For description of some of the foregoin, see, for example, commonly owned Interanational patent applications PCT/US01/23966, PCT/US01/24134, PCTUS/00/41422))

In the methods of the invention, the chimeric polypeptide or nucleic acid that encodes it are employed to induce or enhance immune responses. In one embodiment, the compositions of the invention synergistically enhance immune responses and antitumor effects through both immunological and anti-angiogenic mechanisms.

The experiments described herein demonstrate that the methods of the invention can enhance a cellular immune response, particularly, tumor-destructive CTL reactivity, induced by a DNA vaccine encoding an epitope of a human pathogen. Human HPV-16 E7 was used as a model antigen for vaccine development because human papillomaviruses (HPVs), particularly HPV-16, are associated with most human cervical cancers. The oncogenic HPV protein E7 is important in the induction and maintenance of cellular transformation and co-expressed in most HPV-containing cervical cancers and their precursor

lesions. Therefore, cancer vaccines, such as the compositions of the invention, that target E7 can be used to control of HPV-associated neoplasms (Wu (1994) *Curr. Opin. Immunol.* 6:746-754).

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art of this invention. As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

The term "antigen" or "immunogen" as used herein refers to a compound or composition comprising a peptide, polypeptide or protein which is "antigenic" or "immunogenic" when administered (or expressed *in vivo* by an administered nucleic acid, *e.g.*, a DNA vaccine) in an appropriate amount (an "immunogenically effective amount"), *i.e.*, capable of inducing, eliciting, augmenting or boosting a cellular and/or humoral immune response either alone or in combination or linked or fused to another substance (which can be administered at once or over several intervals). An immunogenic composition can comprise an antigenic peptide of at least about 5 amino acids, a peptide of 10 amino acids in length, a polypepide fragment of 15 amino acids in length, 20 amino acids in length or longer. Smaller immunogens may require presence of a "carrier" polypeptide *e.g.*, as a fusion protein, aggregate, conjugate or mixture, preferablyl linked (chemically or otherwise) to the immunogen. The immunogen can be recombinantly expressed from a vaccine vector, which can be naked DNA comprising the immunogen's coding sequence operably linked to a promoter, *e.g.*, an expression cassette as described herein. The immunogen includes one or more antigenic determinants or epitopes which may vary in size from about 3 to about 15 amino acids.

The term "epitope" as used herein refers to an antigenic determinant or antigenic site that interacts with an antibody or a T cell receptor (TCR), *e.g.*, the MHC class I-binding peptide compositions (or expressed products of the nucleic acid compositionsof the invention) used in the methods of the invention. An "antigen" is a molecule or chemical structure that either induces an immune response or is specifically recognized or bound by the product or mediator of an immune response, such as an antibody or a CTL. The specific conformational or stereochemical "domain" to which an antibody or a TCR bind is an "antigenic determinant" or "epitope." TCRs bind to peptide epitopes which are physically associated with a third molecule, a major histocompatibility complex (MHC) class I or class II protein.

The term "recombinant" refers to (1) a nucleic acid or polynucleotide synthesized or otherwise manipulated *in vitro*, (2) methods of using recombinant DNA technology to produce gene products in cells or other biological systems, or (3) a polypeptide encoded by a recombinant nucleic acid. For example, the FL-encoding nucleic acid or polypeptide, the nucleic acid encoding an MHC class I-binding peptide epitope (antigen) or the peptide itself can be rececombinant. "Recombinant means" includes ligation of nucleic acids having various coding regions or domains or promoter sequences from

different sources into a single unit in the form of an expression cassette or vector for expression of the coding sequences in the vectors resulting in production of the encoded polypeptide.

The term "self-replicating RNA replicon" refers to a construct based on an RNA viruses, such as alphavirus genome RNAs (*e.g.*, Sindbis virus, Semliki Forest virus, *etc.*), that have been engineered to allow expression of heterologous RNAs and proteins. These recombinant vectors are self-replicating ("replicons") which can be introduced into cells as naked RNA or DNA, as described in detail, below. In a preferred embodiment, the self-replicating RNA replicon comprises a Sindbis virus self-replicating RNA vector SINrep5, which is described in detail in U.S. Patent No. 5,217,879.

SEQUENCES OF POLYPEPTIDES AND NUCLEIC ACIDS

The section that follows lists the sequences of the MHC-I-PP and DC-PP polypeptides alone or in fusion with E7 antigen, the nucleic acids encoding some of these peptides and nucleic acids of the vectors into which the sequences encoding these polypeptides are cloned.

HPV-E7 (nucleic acid is SEQ ID NO:1; amino acids are SEQ ID NO:2)

```
1/1                                             31/11
atg cat gga gat aca cct aca ttg cat gaa tat atg tta gat ttg caa cca gag aca act
Met his gly asp thr pro thr leu his glu tyr met leu asp leu gln pro glu thr thr
61/21                                           91/31
gat ctc tac tgt tat gag caa tta aat gac agc tca gag gag gag gat gaa ata gat ggt
asp leu tyr cys tyr glu gln leu asn asp ser ser glu glu glu asp glu ile asp gly
121/41                                          151/51
cca gct gga caa gca gaa ccg gac aga gcc cat tac aat att gta acc ttt tgt tgc aag
pro ala gly gln ala glu pro asp arg ala his tyr asn ile val thr phe cys cys lys
181/61                                          211/71
tgt gac tct acg ctt cgg ttg tgc gta caa agc aca cac gta gac att cgt act ttg gaa
cys asp ser thr leu arg leu cys val gln ser thr his val asp ile arg thr leu glu
241/81                                          271/91
gac ctg tta atg ggc aca cta gga att gtg tgc ccc atc tgt tct cag gat aag ctt
asp leu leu met gly thr leu gly ile val cys pro ile cys ser gln asp lys leu
```

GENBANK Accession No. AAD33353 ("native" sequence). After cloning the last 9 nucleotides (3 amino acids were modified, as shown above underscored and/or bold.

The original GENBANK sequence has a Lys-Pro after the Gln in position 96 (encoded by `aaa/cca/taa`, rather than Asp-Lys-Leu (encoded by `gat/aag/ctt`).

Hsp70 from *M. tuberculosis*

(nucleic acid is SEQ ID NO:3; amino acids are SEQ ID NO:4)

```
1/1                                             31/11
atg gct cgt gcg gtc ggg atc gac ctc ggg acc acc aac tcc gtc gtc tcg gtt ctg gaa
Met ala arg ala val gly ile asp leu gly thr thr asn ser val val ser val leu glu
61/21                                           91/31
ggt ggc gac ccg gtc gtc gtc gcc aac tcc gag ggc tcc agg acc acc ccg tca att gtc
gly gly asp pro val val val ala asn ser glu gly ser arg thr thr pro ser ile val
121/41                                          151/51
gcg ttc gcc cgc aac ggt gag gtg ctg gtc ggc cag ccc gcc aag aac cag gca gtg acc
ala phe ala arg asn gly glu val leu val gly gln pro ala lys asn gln ala val thr
```

```
181/61                                                                  211/71
aac gtc gat cgc acc gtg cgc tcg gtc aag cga cac atg ggc agc gac tgg tcc ata gag
asn val asp arg thr val arg ser val lys arg his met gly ser asp trp ser ile glu
241/81                                                                  271/91
att gac ggc aag aaa tac acc gcg ccg gag atc agc gcc cgc att ctg atg aag ctg aag
ile asp gly lys lys tyr thr ala pro glu ile ser ala arg ile leu met lys leu lys
301/101                                                                 331/111
cgc gac gcc gag gcc tac ctc ggt gag gac att acc gac gcg gtt atc acg acg ccc gcc
arg asp ala glu ala tyr leu gly glu asp ile thr asp ala val ile thr thr pro ala
361/121                                                                 391/131
tac ttc aat gac gcc cag cgt cag gcc acc aag gac gcc ggc cag atc gcc ggc ctc aac
tyr phe asn asp ala gln arg gln ala thr lys asp ala gly gln ile ala gly leu asn
421/141                                                                 451/151
gtg ctg cgg atc gtc aac gag ccg acc gcg gcc gcg ctg gcc tac ggc ctc gac aag ggc
val leu arg ile val asn glu pro thr ala ala ala leu ala tyr gly leu asp lys gly
481/161                                                                 511/171
gag aag gag cag cga atc ctg gtc ttc gac ttg ggt ggt ggc act ttc gac gtt tcc ctg
glu lys glu gln arg ile leu val phe asp leu gly gly gly thr phe asp val ser leu
541/181                                                                 571/191
ctg gag atc ggc gag ggt gtg gtt gag gtc cgt gcc act tcg ggt gac aac cac ctc ggc
leu glu ile gly glu gly val val glu val arg ala thr ser gly asp asn his leu gly
601/201                                                                 631/211
ggc gac gac tgg gac cag cgg gtc gtc gat tgg ctg gtg gac aag ttc aag ggc acc agc
gly asp asp trp asp gln arg val val asp trp leu val asp lys phe lys gly thr ser
661/221                                                                 691/231
ggc atc gat ctg acc aag gac aag atg gcg atg cag cgg ctg cgg gaa gcc gcc gag aag
gly ile asp leu thr lys asp lys met ala met gln arg leu arg glu ala ala glu lys
721/241                                                                 751/251
gca aag atc gag ctg agt tcg agt cag tcc acc tcg atc aac ctg ccc tac atc acc gtc
ala lys ile glu leu ser ser ser gln ser thr ser ile asn leu pro tyr ile thr val
781/261                                                                 811/271
gac gcc gac aag aac ccg ttg ttc tta gac gag cag ctg acc cgc gcg gag ttc caa cgg
asp ala asp lys asn pro leu phe leu asp glu gln leu thr arg ala glu phe gln arg
841/281                                                                 871/291
atc act cag gac ctg ctg gac cgc act cgc aag ccg ttc cag tcg gtg atc gct gac acc
ile thr gln asp leu leu asp arg thr arg lys pro phe gln ser val ile ala asp thr
901/301                                                                 931/311
ggc att tcg gtg tcg gag atc gat cac gtt gtg ctc gtg ggt ggt tcg acc cgg atg ccc
gly ile ser val ser glu ile asp his val val leu val gly gly ser thr arg met pro
961/321                                                                 991/331
gcg gtg acc gat ctg gtc aag gaa ctc acc ggc ggc aag gaa ccc aac aag ggc gtc aac
ala val thr asp leu val lys glu leu thr gly gly lys glu pro asn lys gly val asn
1021/341                                                                1051/351
ccc gat gag gtt gtc gcg gtg gga gcc gct ctg cag gcc ggc gtc ctc aag ggc gag gtg
pro asp glu val val ala val gly ala ala leu gln ala gly val leu lys gly glu val
1081/361                                                                1111/371
aaa gac gtt ctg ctg ctt gat gtt acc ccg ctg agc ctg ggt atc gag acc aag ggc ggg
lys asp val leu leu leu asp val thr pro leu ser leu gly ile glu thr lys gly gly
1141/381                                                                1171/391
gtg atg acc agg ctc atc gag cgc aac acc acg atc ccc acc aag cgg tcg gag act ttc
val met thr arg leu ile glu arg asn thr thr ile pro thr lys arg ser glu thr phe
1201/401                                                                1231/411
acc acc gcc gac gac aac caa ccg tcg gtg cag atc cag gtc tat cag ggg gag cgt gag
thr thr ala asp asp asn gln pro ser val gln ile gln val tyr gln gly glu arg glu
1261/421                                                                1291/431
atc gcc gcg cac aac aag ttg ctc ggg tcc ttc gag ctg acc ggc atc ccg ccg gcg ccg
ile ala ala his asn lys leu leu gly ser phe glu leu thr gly ile pro pro ala pro
1321/441                                                                1351/451
cgg ggg att ccg cag atc gag gtc act ttc gac atc gac gcc aac ggc att gtg cac gtc
arg gly ile pro gln ile glu val thr phe asp ile asp ala asn gly ile val his val
1381/461                                                                1411/471
acc gcc aag gac aag ggc acc ggc aag gag aac acg atc cga atc cag gaa ggc tcg ggc
thr ala lys asp lys gly thr gly lys glu asn thr ile arg ile gln glu gly ser gly
1441/481                                                                1471/491
ctg tcc aag gaa gac att gac cgc atg atc aag gac gcc gaa gcg cac gcc gag gag gat
leu ser lys glu asp ile asp arg met ile lys asp ala glu ala his ala glu glu asp
1501/501                                                                1531/511
cgc aag cgt cgc gag gag gcc gat gtt cgt aat caa gcc gag aca ttg gtc tac cag acg
arg lys arg arg glu glu ala asp val arg asn gln ala glu thr leu val tyr gln thr
1561/521                                                                1591/531
gag aag ttc gtc aaa gaa cag cgt gag gcc gag ggt ggt tcg aag gta cct gaa gac acg
glu lys phe val lys glu gln arg glu ala glu gly gly ser lys val pro glu asp thr   540
1621/541                                                                1651/551
```

```
ctg aac aag gtt gat gcc gcg gtg gcg gaa gcg aag gcg gca ctt ggc gga tcg gat att
leu asn lys val asp ala ala val ala glu ala lys ala ala leu gly gly ser asp ile    560
1681/561                                1711/571
tcg gcc atc aag tcg gcg atg gag aag ctg ggc cag gag tcg cag gct ctg ggg caa gcg
ser ala ile lys ser ala met glu lys leu gly gln glu ser gln ala leu gly gln ala    580
1741/581                                1771/591
atc tac gaa gca gct cag gct gcg tca cag gcc act ggc gct gcc cac ccc ggc tcg gct
ile tyr glu ala ala gln ala ala ser gln ala thr gly ala ala his pro gly ser ala
1801/601
gat gaA AGC
asp glu ser
```

GENBANK Z95324 AL123456; encoded by nucleotides 10633-12510 of *Mycobacterium tuberculosis* genome). As a result of cloning, this has been modified from the original GENBANK sequence which had at its 3' end:

ggc gag ccg ggc ggt gcc cac ccc ggc tcg gct gat gac gtt gtg gac gcg gag gtg gtc gac gac ggc cgg gag gcc aag (SEQ ID NO:5)

which was replaced in the cloned version used herein, by tcg gct gat gaa agc (SEQ ID NO:6) which is bold and underlined above.

E7-Hsp70 Fusion (nucleic acid is SEQ ID NO:7; amino acids are SEQ ID NO:8)

E7 coding sequence is capitalized and underscored.

```
1/1                                     31/11
ATG CAT GGA GAT ACA CCT ACA TTG CAT GAA TAT ATG TTA GAT TTG CAA CCA GAG ACA ACT
Met his gly asp thr pro thr leu his glu tyr met leu asp leu gln pro glu thr thr
61/21                                   91/31
GAT CTC TAC TGT TAT GAG CAA TTA AAT GAC AGC TCA GAG GAG GAG GAT GAA ATA GAT GGT
asp leu tyr cys tyr glu gln leu asn asp ser ser glu glu glu asp glu ile asp gly
121/41                                  151/51
CCA GCT GGA CAA GCA GAA CCG GAC AGA GCC CAT TAC AAT ATT GTA ACC TTT TGT TGC AAG
pro ala gly gln ala glu pro asp arg ala his tyr asn ile val thr phe cys cys lys
181/61                                  211/71
TGT GAC TCT ACG CTT CGG TTG TGC GTA CAA AGC ACA CAC GTA GAC ATT CGT ACT TTG GAA
cys asp ser thr leu arg leu cys val gln ser thr his val asp ile arg thr leu glu
241/81                                  271/91
GAC CTG TTA ATG GGC ACA CTA GGA ATT GTG TGC CCC ATC TGT TCT CAA GGA TCC atg gct
asp leu leu met gly thr leu gly ile val cys pro ile cys ser gln gly ser met ala
301/101                                 331/111
cgt gcg gtc ggg atc gac ctc ggg acc acc aac tcc gtc gtc tcg gtt ctg gaa ggt ggc
arg ala val gly ile asp leu gly thr thr asn ser val val ser val leu glu gly gly
361/121                                 391/131
gac ccg gtc gtc gtc gcc aac tcc gag ggc tcc agg acc acc ccg tca att gtc gcg ttc
asp pro val val val ala asn ser glu gly ser arg thr thr pro ser ile val ala phe
421/141                                 451/151
gcc cgc aac ggt gag gtg ctg gtc ggc cag ccc gcc aag aac cag gca gtg acc aac gtc
ala arg asn gly glu val leu val gly gln pro ala lys asn gln ala val thr asn val
481/161                                 511/171
gat cgc acc gtg cgc tcg gtc aag cga cac atg ggc agc gac tgg tcc ata gag att gac
asp arg thr val arg ser val lys arg his met gly ser asp trp ser ile glu ile asp
541/181                                 571/191
ggc aag aaa tac acc gcg ccg gag atc agc gcc cgc att ctg atg aag ctg aag cgc gac
gly lys lys tyr thr ala pro glu ile ser ala arg ile leu met lys leu lys arg asp
601/201                                 631/211
gcc gag gcc tac ctc ggt gag gac att acc gac gcg gtt atc acg acg ccc gcc tac ttc
ala glu ala tyr leu gly glu asp ile thr asp ala val ile thr thr pro ala tyr phe
661/221                                 691/231
aat gac gcc cag cgt cag gcc acc aag gac gcc ggc cag atc gcc ggc ctc aac gtg ctg
asn asp ala gln arg gln ala thr lys asp ala gly gln ile ala gly leu asn val leu
721/241                                 751/251
cgg atc gtc aac gag ccg acc gcg gcc gcg ctg gcc tac ggc ctc gac aag ggc gag aag
arg ile val asn glu pro thr ala ala ala leu ala tyr gly leu asp lys gly glu lys
781/261                                 811/271
```

```
gag cag cga atc ctg gtc ttc gac ttg ggt ggt ggc act ttc gac gtt tcc ctg ctg gag
glu gln arg ile leu val phe asp leu gly gly gly thr phe asp val ser leu leu glu
841/281                                                 871/291
atc ggc gag ggt gtg gtt gag gtc cgt gcc act tcg ggt gac aac cac ctc ggc ggc gac
ile gly glu gly val val glu val arg ala thr ser gly asp asn his leu gly gly asp
901/301                                                 931/311
gac tgg gac cag cgg gtc gtc gat tgg ctg gtg gac aag ttc aag ggc acc agc ggc atc
asp trp asp gln arg val val asp trp leu val asp lys phe lys gly thr ser gly ile
961/321                                                 991/331
gat ctg acc aag gac aag atg gcg atg cag cgg ctg cgg gaa gcc gcc gag aag gca aag
asp leu thr lys asp lys met ala met gln arg leu arg glu ala ala glu lys ala lys
1021/341                                                1051/351
atc gag ctg agt tcg agt cag tcc acc tcg atc aac ctg ccc tac atc acc gtc gac gcc
ile glu leu ser ser ser gln ser thr ser ile asn leu pro tyr ile thr val asp ala
1081/361                                                1111/371
gac aag aac ccg ttg ttc tta gac gag cag ctg acc cgc gcg gag ttc caa cgg atc act
asp lys asn pro leu phe leu asp glu gln leu thr arg ala glu phe gln arg ile thr
1141/381                                                1171/391
cag gac ctg ctg gac cgc act cgc aag ccg ttc cag tcg gtg atc gct gac acc ggc att
gln asp leu leu asp arg thr arg lys pro phe gln ser val ile ala asp thr gly ile
1201/401                                                1231/411
tcg gtg tcg gag atc gat cac gtt gtg ctc gtg ggt ggt tcg acc cgg atg ccc gcg gtg
ser val ser glu ile asp his val val leu val gly gly ser thr arg met pro ala val
1261/421                                                1291/431
acc gat ctg gtc aag gaa ctc acc ggc ggc aag gaa ccc aac aag ggc gtc aac ccc gat
thr asp leu val lys glu leu thr gly gly lys glu pro asn lys gly val asn pro asp
1321/441                                                1351/451
gag gtt gtc gcg gtg gga gcc gct ctg cag gcc ggc gtc ctc aag ggc gag gtg aaa gac
glu val val ala val gly ala ala leu gln ala gly val leu lys gly glu val lys asp
1381/461                                                1411/471
gtt ctg ctg ctt gat gtt acc ccg ctg agc ctg ggt atc gag acc aag ggc ggg gtg atg
val leu leu leu asp val thr pro leu ser leu gly ile glu thr lys gly gly val met
1441/481                                                1471/491
acc agg ctc atc gag cgc aac acc acg atc ccc acc aag cgg tcg gag act ttc acc acc
thr arg leu ile glu arg asn thr thr ile pro thr lys arg ser glu thr phe thr thr
1501/501                                                1531/511
gcc gac gac aac caa ccg tcg gtg cag atc cag gtc tat cag ggg gag cgt gag atc gcc
ala asp asp asn gln pro ser val gln ile gln val tyr gln gly glu arg glu ile ala
1561/521                                                1591/531
gcg cac aac aag ttg ctc ggg tcc ttc gag ctg acc ggc atc ccg ccg gcg ccg cgg ggg
ala his asn lys leu leu gly ser phe glu leu thr gly ile pro pro ala pro arg gly
1621/541                                                1651/551
att ccg cag atc gag gtc act ttc gac atc gac gcc aac ggc att gtg cac gtc acc gcc
ile pro gln ile glu val thr phe asp ile asp ala asn gly ile val his val thr ala
1681/561                                                1711/571
aag gac aag ggc acc ggc aag gag aac acg atc cga atc cag gaa ggc tcg ggc ctg tcc
lys asp lys gly thr gly lys glu asn thr ile arg ile gln glu gly ser gly leu ser
1741/581                                                1771/591
aag gaa gac att gac cgc atg atc aag gac gcc gaa gcg cac gcc gag gag gat cgc aag
lys glu asp ile asp arg met ile lys asp ala glu ala his ala glu glu asp arg lys
1801/601                                                1831/611
cgt cgc gag gag gcc gat gtt cgt aat caa gcc gag aca ttg gtc tac cag acg gag aag
arg arg glu glu ala asp val arg asn gln ala glu thr leu val tyr gln thr glu lys
1861/621                                                1891/631
ttc gtc aaa gaa cag cgt gag gcc gag ggt ggt tcg aag gta cct gaa gac acg ctg aac
phe val lys glu gln arg glu ala glu gly gly ser lys val pro glu asp thr leu asn
1921/641                                                1951/651
aag gtt gat gcc gcg gtg gcg gaa gcg aag gcg gca ctt ggc gga tcg gat att tcg gcc
lys val asp ala ala val ala glu ala lys ala ala leu gly gly ser asp ile ser ala
1981/661                                                2011/671
atc aag tcg gcg atg gag aag ctg ggc cag gag tcg cag gct ctg ggg caa gcg atc tac
ile lys ser ala met glu lys leu gly gln glu ser gln ala leu gly gln ala ile tyr
2041/681                                                2071/691
gaa gca gct cag gct gcg tca cag gcc act ggc gct gcc cac ccc ggc tcg gct gat gaA
glu ala ala gln ala ala ser gln ala thr gly ala ala his pro gly ser ala asp glu
2101/701
AGC a
ser
```

Flt3 Ligand (FL) extracellular domain

(nucleic acid is SEQ ID NO:9; amino acids are SEQ ID NO:10)

```
1/1                                                 31/11
atg aca gtg ctg gcg cca gcc tgg agc cca aat tcc tcc ctg ttg ctg ctg ttg ctg ctg
Met thr val leu ala pro ala trp ser pro asn ser ser leu leu leu leu leu leu leu
61/21                                               91/31
ctg agt cct tgc ctg cgg ggg aca cct gac tgt tac ttc agc cac agt ccc atc tcc tcc
leu ser pro cys leu arg gly thr pro asp cys tyr phe ser his ser pro ile ser ser
121/41                                              151/51
aac ttc aaa gtg aag ttt aga gag ttg act gac cac ctg ctt aaa gat tac cca gtc act
asn phe lys val lys phe arg glu leu thr asp his leu leu lys asp tyr pro val thr
181/61                                              211/71
gtg gcc gtc aat ctt cag gac gag aag cac tgc aag gcc ttg tgg agc ctc ttc cta gcc
val ala val asn leu gln asp glu lys his cys lys ala leu trp ser leu phe leu ala
241/81                                              271/91
cag cgc tgg ata gag caa ctg aag act gtg gca ggg tct aag atg caa acg ctt ctg gag
gln arg trp ile glu gln leu lys thr val ala gly ser lys met gln thr leu leu glu
301/101                                             331/111
gac gtc aac acc gag ata cat ttt gtc acc tca tgt acc ttc cag ccc cta cca gaa tgt
asp val asn thr glu ile his phe val thr ser cys thr phe gln pro leu pro glu cys
361/121                                             391/131
ctg cga ttc gtc cag acc aac atc tcc cac ctc ctg aag gac acc tgc aca cag ctg ctt
leu arg phe val gln thr asn ile ser his leu leu lys asp thr cys thr gln leu leu
421/141                                             451/151
gct ctg aag ccc tgt atc ggg aag gcc tgc cag aat ttc tct cgg tgc ctg gag gtg cag
ala leu lys pro cys ile gly lys ala cys gln asn phe ser arg cys leu glu val gln
481/161                                             511/171
tgc cag ccg gac tcc tcc acc ctg ctg ccc cca agg agt ccc ata gcc cta gaa gcc acg
cys gln pro asp ser ser thr leu leu pro pro arg ser pro ile ala leu glu ala thr
541/181
gag ctc cca gag cct cgg ccc agg cag
glu leu pro glu pro arg pro arg gln
```

FL-E7 Fusion Polypeptide

(nucleic acid is SEQ ID NO:11; amino acids are SEQ ID NO:12)

N-terminal sequence is FL, followed by E7 (underscored, cap)

```
1/1                                                 31/11
atg aca gtg ctg gcg cca gcc tgg agc cca aat tcc tcc ctg ttg ctg ctg ttg ctg ctg
Met thr val leu ala pro ala trp ser pro asn ser ser leu leu leu leu leu leu leu
61/21                                               91/31
ctg agt cct tgc ctg cgg ggg aca cct gac tgt tac ttc agc cac agt ccc atc tcc tcc
leu ser pro cys leu arg gly thr pro asp cys tyr phe ser his ser pro ile ser ser
121/41                                              151/51
aac ttc aaa gtg aag ttt aga gag ttg act gac cac ctg ctt aaa gat tac cca gtc act
asn phe lys val lys phe arg glu leu thr asp his leu leu lys asp tyr pro val thr
181/61                                              211/71
gtg gcc gtc aat ctt cag gac gag aag cac tgc aag gcc ttg tgg agc ctc ttc cta gcc
val ala val asn leu gln asp glu lys his cys lys ala leu trp ser leu phe leu ala
241/81                                              271/91
cag cgc tgg ata gag caa ctg aag act gtg gca ggg tct aag atg caa acg ctt ctg gag
gln arg trp ile glu gln leu lys thr val ala gly ser lys met gln thr leu leu glu
301/101                                             331/111
gac gtc aac acc gag ata cat ttt gtc acc tca tgt acc ttc cag ccc cta cca gaa tgt
asp val asn thr glu ile his phe val thr ser cys thr phe gln pro leu pro glu cys
361/121                                             391/131
ctg cga ttc gtc cag acc aac atc tcc cac ctc ctg aag gac acc tgc aca cag ctg ctt
leu arg phe val gln thr asn ile ser his leu leu lys asp thr cys thr gln leu leu
421/141                                             451/151
gct ctg aag ccc tgt atc ggg aag gcc tgc cag aat ttc tct cgg tgc ctg gag gtg cag
ala leu lys pro cys ile gly lys ala cys gln asn phe ser arg cys leu glu val gln
481/161                                             511/171
tgc cag ccg gac tcc tcc acc ctg ctg ccc cca agg agt ccc ata gcc cta gaa gcc acg
cys gln pro asp ser ser thr leu leu pro pro arg ser pro ile ala leu glu ala thr
541/181                                             571/191
gag ctc cca gag cct cgg ccc agg cag gaa ttc ATG CAT GGA GAT ACA CCT ACA TTG CAT
glu leu pro glu pro arg pro arg gln glu phe met his gly asp thr pro thr leu his
```

601/201 631/211
GAA TAT ATG TTA GAT TTG CAA CCA GAG ACA ACT GAT CTC TAC TGT TAT GAG CAA TTA AAT
glu tyr met leu asp leu gln pro glu thr thr asp leu tyr cys tyr glu gln leu asn
661/221 691/231
GAC AGC TCA GAG GAG GAG GAT GAA ATA GAT GGT CCA GCT GGA CAA GCA GAA CCG GAC AGA
asp ser ser glu glu glu asp glu ile asp gly pro ala gly gln ala glu pro asp arg
721/241 751/251
GCC CAT TAC AAT ATT GTA ACC TTT TGT TGC AAG TGT GAC TCT ACG CTT CGG TTG TGC GTA
ala his tyr asn ile val thr phe cys cys lys cys asp ser thr leu arg leu cys val
781/261 811/271
CAA AGC ACA CAC GTA GAC ATT CGT ACT TTG GAA GAC CTG TTA ATG GGC ACA CTA GGA ATT
gln ser thr his val asp ile arg thr leu glu asp leu leu met gly thr leu gly ile
841/281
GTG TGC CCC ATC TGT TCT CAA GGA TCC
val cys pro ile cys ser gln gly ser

FL-E7-GFP Fusion Polypeptide

(nucleic acid is SEQ ID NO:13; amino acids are SEQ ID NO:14)

N-terminal sequence is FL, followed by E7 (underscored, cap) followed by GFP (italic)

1/1 31/11
atg aca gtg ctg gcg cca gcc tgg agc cca aat tcc tcc ctg ttg ctg ctg ttg ctg ctg
Met thr val leu ala pro ala trp ser pro asn ser ser leu leu leu leu leu leu leu
61/21 91/31
ctg agt cct tgc ctg cgg ggg aca cct gac tgt tac ttc agc cac agt ccc atc tcc tcc
leu ser pro cys leu arg gly thr pro asp cys tyr phe ser his ser pro ile ser ser
121/41 151/51
aac ttc aaa gtg aag ttt aga gag ttg act gac cac ctg ctt aaa gat tac cca gtc act
asn phe lys val lys phe arg glu leu thr asp his leu leu lys asp tyr pro val thr
181/61 211/71
gtg gcc gtc aat ctt cag gac gag aag cac tgc aag gcc ttg tgg agc ctc ttc cta gcc
val ala val asn leu gln asp glu lys his cys lys ala leu trp ser leu phe leu ala
241/81 271/91
cag cgc tgg ata gag caa ctg aag act gtg gca ggg tct aag atg caa acg ctt ctg gag
gln arg trp ile glu gln leu lys thr val ala gly ser lys met gln thr leu leu glu
301/101 331/111
gac gtc aac acc gag ata cat ttt gtc acc tca tgt acc ttc cag ccc cta cca gaa tgt
asp val asn thr glu ile his phe val thr ser cys thr phe gln pro leu pro glu cys
361/121 391/131
ctg cga ttc gtc cag acc aac atc tcc cac ctc ctg aag gac acc tgc aca cag ctg ctt
leu arg phe val gln thr asn ile ser his leu leu lys asp thr cys thr gln leu leu
421/141 451/151
gct ctg aag ccc tgt atc ggg aag gcc tgc cag aat ttc tct cgg tgc ctg gag gtg cag
ala leu lys pro cys ile gly lys ala cys gln asn phe ser arg cys leu glu val gln
481/161 511/171
tgc cag ccg gac tcc tcc acc ctg ctg ccc cca agg agt ccc ata gcc cta gaa gcc acg
cys gln pro asp ser ser thr leu leu pro pro arg ser pro ile ala leu glu ala thr
541/181 571/191
gag ctc cca gag cct cgg ccc agg cag gaa ttc ATG CAT GGA GAT ACA CCT ACA TTG CAT
glu leu pro glu pro arg pro arg gln glu phe met his gly asp thr pro thr leu his
601/201 631/211
GAA TAT ATG TTA GAT TTG CAA CCA GAG ACA ACT GAT CTC TAC TGT TAT GAG CAA TTA AAT
glu tyr met leu asp leu gln pro glu thr thr asp leu tyr cys tyr glu gln leu asn
661/221 691/231
GAC AGC TCA GAG GAG GAG GAT GAA ATA GAT GGT CCA GCT GGA CAA GCA GAA CCG GAC AGA
asp ser ser glu glu glu asp glu ile asp gly pro ala gly gln ala glu pro asp arg
721/241 751/251
GCC CAT TAC AAT ATT GTA ACC TTT TGT TGC AAG TGT GAC TCT ACG CTT CGG TTG TGC GTA
ala his tyr asn ile val thr phe cys cys lys cys asp ser thr leu arg leu cys val
781/261 811/271
CAA AGC ACA CAC GTA GAC ATT CGT ACT TTG GAA GAC CTG TTA ATG GGC ACA CTA GGA ATT
gln ser thr his val asp ile arg thr leu glu asp leu leu met gly thr leu gly ile
841/281 871/291
GTG TGC CCC ATC TGT TCT CAA GGA TCC *atg gtg agc aag ggc gag gag ctg ttc acc ggg*
val cys pro ile cys ser gln gly ser *met val ser lys gly glu glu leu phe thr gly*
901/301 931/311
gtg gtg ccc atc ctg gtc gag ctg gac ggc gac gta aac ggc cac aag ttc agc gtg tcc
val val pro ile leu val glu leu asp gly asp val asn gly his lys phe ser val ser
961/321 991/331

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ggc gag ggc gag ggc gat gcc acc tac ggc aag ctg acc ctg aag ttc atc tgc acc acc
gly glu gly glu gly asp ala thr tyr gly lys leu thr leu lys phe ile cys thr thr
1021/341                                                    1051/351
ggc aag ctg ccc gtg ccc tgg ccc acc ctc gtg acc acc ttc acc tac ggc gtg cag tgc
gly lys leu pro val pro trp pro thr leu val thr thr phe thr tyr gly val gln cys
1081/361                                                    1111/371
ttc agc cgc tac ccc gac cac atg aag cag cac gac ttc ttc aag tcc gcc atg ccc gaa
phe ser arg tyr pro asp his met lys gln his asp phe phe lys ser ala met pro glu
1141/381                                                    1171/391
ggc tac gtc cag gag cgc acc atc ttc ttc aag gac gac ggc aac tac aag acc cgc gcc
gly tyr val gln glu arg thr ile phe phe lys asp asp gly asn tyr lys thr arg ala
1201/401                                                    1231/411
gag gtg aag ttc gag ggc gac acc ctg gtg aac cgc atc gag ctg aag ggc atc gac ttc
glu val lys phe glu gly asp thr leu val asn arg ile glu leu lys gly ile asp phe
1261/421                                                    1291/431
aag gag gac ggc aac atc ctg ggg cac aag ctg gag tac aac tac aac agc cac aac gtc
lys glu asp gly asn ile leu gly his lys leu glu tyr asn tyr asn ser his asn val
1321/441                                                    1351/451
tat atc atg gcc gac aag cag aag aac ggc atc aag gtg aac ttc aag atc cgc cac aac
tyr ile met ala asp lys gln lys asn gly ile lys val asn phe lys ile arg his asn
1381/461                                                    1411/471
atc gag gac ggc agc gtg cag ctc gcc gac cac tac cag cag aac acc ccc atc ggc gac
ile glu asp gly ser val gln leu ala asp his tyr gln gln asn thr pro ile gly asp
1441/481                                                    1471/491
ggc ccc gtg ctg ctg ccc gac aac cac tac ctg agc acc cag tcc gcc ctg agc aaa gac
gly pro val leu leu pro asp asn his tyr leu ser thr gln ser ala leu ser lys asp
1501/501                                                    1531/511
ccc aac gag aag cgc gat cac atg gtc ctg ctg gag ttc gtg acc gcc gcc ggg atc act
pro asn glu lys arg asp his met val leu leu glu phe val thr ala ala gly ile thr
1561/521
ctc ggc atg gac gag ctg tac aag
leu gly met asp glu leu tyr lys
```

pcDNA3 plasmid vector: (SEQ ID NO:15)

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GACGGATCGG GAGATCTCCC GATCCCCTAT GGTCGACTCT CAGTACAATC TGCTCTGATG CCGCATAGTT
AAGCCAGTAT CTGCTCCCTG CTTGTGTGTT GGAGGTCGCT GAGTAGTGCG CGAGCAAAAT TTAAGCTACA
ACAAGGCAAG GCTTGACCGA CAATTGCATG AAGAATCTGC TTAGGGTTAG GCGTTTTGCG CTGCTTCGCG
ATGTACGGGC CAGATATACG CGTTGACATT GATTATTGAC TAGTTATTAA TAGTAATCAA TTACGGGGTC
ATTAGTTCAT AGCCCATATA TGGAGTTCCG CGTTACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG
CCCAACGACC CCCGCCCATT GACGTCAATA ATGACGTATG TTCCCATAGT AACGCCAATA GGGACTTTCC
ATTGACGTCA ATGGGTGGAC TATTTACGGT AAACTGCCCA CTTGGCAGTA CATCAAGTGT ATCATATGCC
AAGTACGCCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT ATGCCCAGTA CATGACCTTA
TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA TCGCTATTAC CATGGTGATG CGGTTTTGGC
AGTACATCAA TGGGCGTGGA TAGCGGTTTG ACTCACGGGG ATTTCCAAGT CTCCACCCCA TTGACGTCAA
TGGGAGTTTG TTTTGGCACC AAAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG
CAAATGGGCG GTAGGCGTGT ACGGTGGGAG GTCTATATAA GCAGAGCTCT CTGGCTAACT AGAGAACCCA
CTGCTTACTG GCTTATCGAA ATTAATACGA CTCACTATAG GGAGACCCAA GCTGGCTAGC GTTTAAACGG
GCCCTCTAGA CTCGAGCGGC CGCCACTGTG CTGGATATCT GCAGAATTCC ACCACACTGG ACTAGTGGAT
CCGAGCTCGG TACCAAGCTT AAGTTTAAAC CGCTGATCAG CCTCGACTGT GCCTTCTAGT TGCCAGCCAT
CTGTTGTTTG CCCCTCCCCC GTGCCTTCCT TGACCCTGGA AGGTGCCACT CCCACTGTCC TTTCCTAATA
AAATGAGGAA ATTGCATCGC ATTGTCTGAG TAGGTGTCAT TCTATTCTGG GGGGTGGGGT GGGGCAGGAC
AGCAAGGGGG AGGATTGGGA AGACAATAGC AGGCATGCTG GGGATGCGGT GGGCTCTATG GCTTCTGAGG
CGGAAAGAAC CAGCTGGGGC TCTAGGGGGT ATCCCCACGC GCCCTGTAGC GGCGCATTAA GCGCGGCGGG
TGTGGTGGTT ACGCGCAGCG TGACCGCTAC ACTTGCCAGC GCCCTAGCGC CCGCTCCTTT CGCTTTCTTC
CCTTCCTTTC TCGCCACGTT CGCCGGCTTT CCCCGTCAAG CTCTAAATCG GGGCATCCCT TTAGGGTTCC
GATTTAGTGC TTTACGGCAC CTCGACCCCA AAAAACTTGA TTAGGGTGAT GGTTCACGTA GTGGGCCATC
GCCCTGATAG ACGGTTTTTC GCCCTTTGAC GTTGGAGTCC ACGTTCTTTA ATAGTGGACT CTTGTTCCAA
ACTGGAACAA CACTCAACCC TATCTCGGTC TATTCTTTTG ATTTATAAGG GATTTTGGGG ATTTCGGCCT
ATTGGTTAAA AAATGAGCTG ATTTAACAAA AATTTAACGC GAATTAATTC TGTGGAATGT GTGTCAGTTA
GGGTGTGGAA AGTCCCCAGG CTCCCCAGGC AGGCAGAAGT ATGCAAAGCA TGCATCTCAA TTAGTCAGCA
ACCAGGTGTG GAAAGTCCCC AGGCTCCCCA GCAGGCAGAA GTATGCAAAG CATGCATCTC AATTAGTCAG
CAACCATAGT CCCGCCCCTA ACTCCGCCCA TCCCGCCCCT AACTCCGCCC AGTTCCGCCC ATTCTCCGCC
CCATGGCTGA CTAATTTTTT TTATTTATGC AGAGGCCGAG GCCGCCTCTG CCTCTGAGCT ATTCCAGAAG
TAGTGAGGAG GCTTTTTTGG AGGCCTAGGC TTTTGCAAAA AGCTCCCGGG AGCTTGTATA TCCATTTTCG
GATCTGATCA AGAGACAGGA TGAGGATCGT TTCGCATGAT TGAACAAGAT GGATTGCACG CAGGTTCTCC
GGCCGCTTGG GTGGAGAGGC TATTCGGCTA TGACTGGGCA CAACAGACAA TCGGCTGCTC TGATGCCGCC
GTGTTCCGGC TGTCAGCGCA GGGGCGCCCG GTTCTTTTTG TCAAGACCGA CCTGTCCGGT GCCCTGAATG
AACTGCAGGA CGAGGCAGCG CGGCTATCGT GGCTGGCCAC GACGGGCGTT CCTTGCGCAG CTGTGCTCGA
CGTTGTCACT GAAGCGGGAA GGGACTGGCT GCTATTGGGC GAAGTGCCGG GGCAGGATCT CCTGTCATCT
CACCTTGCTC CTGCCGAGAA AGTATCCATC ATGGCTGATG CAATGCGGCG GCTGCATACG CTTGATCCGG
CTACCTGCCC ATTCGACCAC CAAGCGAAAC ATCGCATCGA GCGAGCACGT ACTCGGATGG AAGCCGGTCT
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TGTCGATCAG GATGATCTGG ACGAAGAGCA TCAGGGGCTC GCGCCAGCCG AACTGTTCGC CAGGCTCAAG
GCGCGCATGC CCGACGGCGA GGATCTCGTC GTGACCCATG GCGATGCCTG CTTGCCGAAT ATCATGGTGG
AAAATGGCCG CTTTTCTGGA TTCATCGACT GTGGCCGGCT GGGTGTGGCG GACCGCTATC AGGACATAGC
GTTGGCTACC CGTGATATTG CTGAAGAGCT TGGCGGCGAA TGGGCTGACC GCTTCCTCGT GCTTTACGGT
ATCGCCGCTC CCGATTCGCA GCGCATCGCC TTCTATCGCC TTCTTGACGA GTTCTTCTGA GCGGGACTCT
GGGGTTCGAA ATGACCGACC AAGCGACGCC CAACCTGCCA TCACGAGATT TCGATTCCAC CGCCGCCTTC
TATGAAAGGT TGGGCTTCGG AATCGTTTTC CGGGACGCCG GCTGGATGAT CCTCCAGCGC GGGGATCTCA
TGCTGGAGTT CTTCGCCCAC CCCAACTTGT TTATTGCAGC TTATAATGGT TACAAATAAA GCAATAGCAT
CACAAATTTC ACAAATAAAG CATTTTTTTC ACTGCATTCT AGTTGTGGTT TGTCCAAACT CATCAATGTA
TCTTATCATG TCTGTATACC GTCGACCTCT AGCTAGAGCT TGGCGTAATC ATGGTCATAG CTGTTTCCTG
TGTGAAATTG TTATCCGCTC ACAATTCCAC ACAACATACG AGCCGGAAGC ATAAAGTGTA AAGCCTGGGG
TGCCTAATGA GTGAGCTAAC TCACATTAAT TGCGTTGCGC TCACTGCCCG CTTTCCAGTC GGGAAACCTG
TCGTGCCAGC TGCATTAATG AATCGGCCAA CGCGCGGGGA GAGGCGGTTT GCGTATTGGG CGCTCTTCCG
CTTCCTCGCT CACTGACTCG CTGCGCTCGG TCGTTCGGCT GCGGCGAGCG GTATCAGCTC ACTCAAAGGC
GGTAATACGG TTATCCACAG AATCAGGGGA TAACGCAGGA AAGAACATGT GAGCAAAAGG CCAGCAAAAG
GCCAGGAACC GTAAAAAGGC CGCGTTGCTG GCGTTTTTCC ATAGGCTCCG CCCCCCTGAC GAGCATCACA
AAAATCGACG CTCAAGTCAG AGGTGGCGAA ACCCGACAGG ACTATAAAGA TACCAGGCGT TTCCCCCTGG
AAGCTCCCTC GTGCGCTCTC CTGTTCCGAC CCTGCCGCTT ACCGGATACC TGTCCGCCTT TCTCCCTTCG
GGAAGCGTGG CGCTTTCTCA ATGCTCACGC TGTAGGTATC TCAGTTCGGT GTAGGTCGTT CGCTCCAAGC
TGGGCTGTGT GCACGAACCC CCCGTTCAGC CCGACCGCTG CGCCTTATCC GGTAACTATC GTCTTGAGTC
CAACCCGGTA AGACACGACT TATCGCCACT GGCAGCAGCC ACTGGTAACA GGATTAGCAG AGCGAGGTAT
GTAGGCGGTG CTACAGAGTT CTTGAAGTGG TGGCCTAACT ACGGCTACAC TAGAAGGACA GTATTTGGTA
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pcDNA3-E7-Hsp70 SEQ ID NO:18 The E7-Hsp70 fusion sequence is shown in bold, caps

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7041  gccatccgta agatgctttt ctgtgactgg tgagtactca accaagtcat tctgagaata gtgtatgcgg cgaccgagtt  7120
7121  gctcttgccc ggcgtcaata cgggataata ccgcgccaca tagcagaact ttaaaagtgc tcatcattgg aaaacgttct  7200
7201  tcggggcgaa aactctcaag gatcttaccg ctgttgagat ccagttcgat gtaacccact cgtgcaccca actgatcttc  7280
7281  agcatctttt actttcacca gcgtttctgg gtgagcaaaa acaggaaggc aaaatgccgc aaaaaaggga ataagggcga  7360
7361  cacggaaatg ttgaatactc atactcttcc tttttcaata ttattgaagc atttatcagg gttattgtct catgagcgga  440
7441  tacatatttg aatgtattta gaaaaataaa caaatagggg ttccgcgcac atttccccga aaagtgccac ctgacgtc    7518
      |  10  |  20  |  30  |  40  |  50  |  60  |  70  |  80  |
```

SINrep5-E7-Hsp70 SEQ ID NO:19 The E7-Hsp70 fusion sequence is shown in bold, caps

```
      |  10  |  20  |  30  |  40  |  50  |  60  |  70  |  80  |
    1 attgacggcg tagtacacac tattgaatca aacagccgac caattgcact accatcacaa tggagaagcc agtagtaaac   80
   81 gtagacgtag acccccagag tccgtttgtc gtgcaactgc aaaaaagctt cccgcaattt gaggtagtag cacagcaggt  160
  161 cactccaaat gaccatgcta atgccagagc attttcgcat ctggccagta aactaatcga gctggaggtt cctaccacag  240
  241 cgacgatctt ggacataggc agcgcaccgg ctcgtagaat gttttccgag caccagtatc attgtgtctg ccccatgcgt  320
  321 agtccagaag acccggaccg catgatgaaa tacgccagta aactggcgga aaaagcgtgc aagattacaa acaagaactt  400
  401 gcatgagaag attaaggatc tccggaccgt acttgatacg ccggatgctg aaacaccatc gctctgcttt cacaacgatg  480
  481 ttacctgcaa catgcgtgcc gaatattccg tcatgcagga cgtgtatatc aacgctcccg gaactatcta tcatcaggct  560
  561 atgaaaggcg tgcggaccct gtactggatt ggcttcgaca ccacccagtt catgttctcg gctatggcag gttcgtaccc  640
  641 tgcgtacaac accaactggg ccgacgagaa agtccttgaa gcgcgtaaca tcggactttg cagcacaaag ctgagtgaag  720
  721 gtaggacagg aaaattgtcg ataatgagga agaaggagtt gaagcccggg tcgcgggttt atttctccgt aggatcgaca  800
  801 ctttatccag aacacagagc cagcttgcag agctggcatc ttccatcggt gttccacttg aatggaaagc agtcgtacac  880
  881 ttgccgctgt gatacagtgg tgagttgcga aggctacgta gtgaagaaaa tcaccatcag tcccgggatc acgggagaaa  960
  961 ccgtgggata cgcggttaca cacaatagcg agggcttctt gctatgcaaa gttactgaca cagtaaaagg agaacgggta 1040
 1041 tcgttccctg tgtgcacgta catcccggcc accatatgcg atcagatgac tggtataatg gccacggata tatcacctga 1120
 1121 cgatgcacaa aaacttctgg ttgggctcaa ccagcgaatt gtcattaacg gtaggactaa caggaacacc aacaccatgc 1200
 1201 aaaattacct tctgccgatc atagcacaag ggttcagcaa atgggctaag gagcgcaagg atgatcttga taacgagaaa 1280
 1281 atgctgggta ctagagaacg caagcttacg tatggctgct tgtgggcgtt tcgcactaag aaagtacatt cgttttatcg 1360
 1361 cccacctgga acgcagacct gcgtaaaagt cccagcctct tttagcgctt ttcccatgtc gtccgtatgg acgacctctt 1440
 1441 tgcccatgtc gctgaggcag aaattgaaac tggcattgca accaaagaag gaggaaaaac tgctgcaggt ctcggaggaa 1520
 1521 ttagtcatgg aggccaaggc tgcttttgag gatgctcagg aggaagccag agcggagaag ctccgagaag cacttccacc 1600
 1601 attagtggca gacaaaggca tcgaggcagc cgcagaagtt gtctgcgaag tggaggggct ccaggcggac atcggagcag 1680
 1681 cattagttga aaccccgcgc ggtcacgtaa ggataatacc tcaagcaaat gaccgtatga tcggacagta tatcgttgtc 1760
 1761 tcgccaaact ctgtgctgaa gaatgccaaa ctcgcaccag cgcacccgct agcagatcag gttaagatca taacacactc 1840
 1841 cggaagatca ggaaggtacg cggtcgaacc atacgacgct aaagtactga tgccagcagg aggtgccgta ccatggccag 1920
 1921 aattcctagc actgagtgag agcgccacgt tagtgtacaa cgaaagagag tttgtgaacc gcaaactata ccacattgcc 2000
```

```
2001 atgcatggcc ccgccaagaa tacagaagag gagcagtaca aggttacaaa ggcagagctt gcagaaacag agtacgtgtt 2080
2081 tgacgtggac aagaagcgtt gcgttaagaa ggaagaagcc tcaggtctgg tcctctcggg agaactgacc aaccctccct 2160
2161 atcatgagct agctctggag ggactgaaga cccgacctgc ggtcccgtac aaggtcgaaa caataggagt gataggcaca 2240
2241 ccgggtcgg gcaagtcagc tattatcaag tcaactgtca cggcacgaga tcttgttacc agcggaaaga aagaaaattg 2320
2321 tcgcgaaatt gaggccgacg tgctaagact gaggggtatg cagattacgt cgaagacagt agattcggtt atgctcaacg 2400
2401 gatgccacaa agccgtagaa gtgctgtacg ttgacgaagc gttcgcgtgc cacgcaggag cactacttgc cttgattgct 2480
2481 atcgtcaggc cccgcaagaa ggtagtacta tgcggagacc ccatgcaatg cggattcttc aacatgatgc aactaaaggt 2560
2561 acatttcaat cacctgaaa aagacatatg caccaagaca ttctacaagt atatctcccg gcgttgcaca cagccagtta 2640
2641 cagctattgt atcgacactg cattacgatg gaaagatgaa aaccacgaac ccgtgcaaga agaacattga aatcgatatt 2720
2721 acagggccca caaagccgaa gccagggat atcatcctga catgtttccg cgggtgggtt aagcaattgc aaatcgacta 2800
2801 tcccggacat gaagtaatga cagccgcggc ctcacaagga ctaaccagaa aaggagtgta tgccgtccgg caaaaagtca 2880
2881 atgaaaaccc actgtacgcg atcacatcag agcatgtgaa cgtgttgctc acccgcactg aggacaggct agtgtggaaa 2960
2961 accttgcagg gcgacccatg gattaagcag cccactaaca tacctaaagg aaactttcag gctactatag aggactggga 3040
3041 agctgaacac aagggaataa ttgctgcaat aaacagcccc actccccgtg ccaatccgtt cagctgcaag accaacgttt 3120
3121 gctgggcgaa agcattggaa ccgatactag ccacggccgg tatcgtactt accggttgcc agtggagcga actgttccca 3200
3201 cagtttgcgg atgacaaacc acattcggcc atttacgcct tagacgtaat ttgcattaag ttttcggca tggacttgac 3280
3281 aagcggactg tttctaaac agagcatccc actaacgtac catcccgccg attcagcgag gccggtagct cattgggaca 3360
3361 acagcccagg aacccgcaag tatgggtacg atcacgccat tgccgccgaa ctctcccgta gatttccggt gttccagcta 3440
3441 gctgggaagg gcacacaact tgatttgcag acggggagaa ccagagttat ctctgcacag cataacctgg tcccggtgaa 3520
3521 ccgcaatctt cctcacgcct tagtcccccga gtacaaggag aagcaacccg gcccggtcaa aaaattcttg aaccagttca 3600
3601 aacaccactc agtacttgtg gtatcagagg aaaaaattga agctccccgt aagagaatcg aatggatcgc cccgattggc 3680
3681 atagccggtg cagataagaa ctacaacctg gctttcgggt ttccgccgca ggcacggtac gacctggtgt tcatcaacat 3760
3761 tggaactaaa tacagaaacc accactttca gcagtgcgaa gaccatgcgg cgaccttaaa aaccctttcg cgttcggccc 3840
3841 tgaattgcct taacccagga ggcaccctcg tggtgaagtc ctatggctac gccgaccgca acagtgagga cgtagtcacc 3920
3921 gctcttgcca gaaagtttgt cagggtgtct gcagcgagac cagattgtgt ctcaagcaat acagaaatgt acctgatttt 4000
4001 ccgacaacta gacaacagcc gtacacggca attcacccag caccatctga attgcgtgat ttcgtccgtg tatgagggta 4080
4081 caagagatgg agttggagcc gcgccgtcat accgcaccaa aagggagaat attgctgact gtcaagagga agcagttgtc 4160
4161 aacgcagcca atccgctggg tagaccaggc gaaggagtct gccgtgccat ctataaacgt tggccgacca gttttaccga 4240
4241 ttcagccacg gagacaggca ccgcaagaat gactgtgtgc ctaggaaaga aagtgatcca cgcggtcggc cctgatttcc 4320
4321 ggaagcaccc agaagcagaa gccttgaaat tgctacaaaa cgcctaccat gcagtggcag acttagtaaa tgaacataac 4400
4401 atcaagtctg tcgccattcc actgctatct acgcagccgg aaaagaccgc cttgaagtat cacttaactg 4480
4481 cttgacaacc gcgctagaca gaactgacgc ggacgtaacc atctattgcc tggataagaa gtggaaggaa agaatcgacg 4560
4561 cggcactcca acttaaggag tctgtaacag agctgaagga tgaagatatg gagatcgacg atgagttagt atggattcat 4640
4641 ccagacagtt gcttgaaggg aagaaaggga ttcagtacta caaaaggaaa attgtattcg tacttcgaag gcaccaaatt 4720
4721 ccatcaagca gcaaaagaca tggcggagat aaagtcctg ttccctaatg accaggaaag taatgaacaa ctgtgtgcct 4800
4801 acatattggg tgagaccatg gaagcaatcc gcgaaaagtg cccggtcgac cataacccgt cgtctagccc gcccaaaacg 4880
4881 ttgccgtgcc ttgcatgtga tgccatgacg ccagaaaggg tccacagact tagaagcaat aacgtcaaag aagttacagt 4960
4961 atgctcctcc acccccttc ctaagcacaa aattaagaat gttcagaagg ttcagtgcac gaaagtagtc ctgtttaatc 5040
5041 cgcacactcc cgcattcgtt cccgcccgta agtacataga agtgccagaa cagcctaccg ctcctcctgc acaggccgag 5120
5121 gaggccccgg aagttgtagc gacaccgtca ccatctacag ctgataacac ctcgcttgat gtcacagaca tctcactgga 5200
5201 tatggatgac agtagcgaag gctcactttt ttcgagcttt agcggatcgg acaactctat tactagtatg gacagttggt 5280
5281 cgtcaggacc tagttcacta gagatagtag accgaaggca ggtggtggtg gctgacgttc atgccgtcca agagcctgcc 5360
5361 cctattccac cgccaaggct aaagaagatg gcccgcctgg cagcggcaag aaaagagccc actccaccgg caagcaatag 5440
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5441 ctctgagtcc ctccacctct ctttggtgg ggtatccatg tccctcggat caattttcga cggagagacg gcccgccagg 5520
5521 cagcggtaca acccctggca acaggcccca cggatgtgcc tatgtctttc ggatcgtttt ccgacggaga gattgatgag 5600
5601 ctgagccgca gagtaactga gtccgaaccc gtcctgtttg gatcatttga accgggcgaa gtgaactcaa ttatatcgtc 5680
5681 ccgatcagcc gtatcttttc cactacgcaa gcagagacgt agacgcagga gcaggaggac tgaatactga ctaaccgggg 5760
5761 taggtgggta catattttcg acggacacag gccctgggca cttgcaaaag aagtccgttc tgcagaacca gcttacagaa 5840
5841 ccgaccttgg agcgcaatgt cctggaaaga attcatgccc cggtgctcga cacgtcgaaa gaggaacaac tcaaactcag 5920
5921 gtaccagatg atgcccaccg aagccaacaa aagtaggtac cagtctcgta aagtagaaaa tcagaaagcc ataaccactg 6000
6001 agcgactact gtcaggacta cgactgtata actctgccac agatcagcca gaatgctata agatcaccta tccgaaacca 6080
6081 ttgtactcca gtagcgtacc ggcgaactac tccgatccac agttcgctgt agctgtctgt aacaactatc tgcatgagaa 6160
6161 ctatccgaca gtagcatctt atcagattac tgacgagtac gatgcttact tggatatggt agacgggaca gtcgcctgcc 6240
6241 tggatactgc aaccttctgc cccgctaagc ttagaagtta cccgaaaaaa catgagtata gagccccgaa tatccgcagt 6320
6321 gcggttccat cagcgatgca gaacacgcta caaaatgtgc tcattgccgc aactaaaaga aattgcaacg tcacgcagat 6400
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6561 gccgcactat ttgcaaagac gtataatttg gtcccattgc aagaagtgcc tatggataga ttcgtcatgg acatgaaaag 6640
6641 agacgtgaaa gttacaccag gcacgaaaca cacagaagaa agaccgaaag tacaagtgat acaagccgca gaaccctgg 6720
6721 cgactgctta cttatcgggg attcaccggg aattagtgcg taggcttacg gccgtcttgc ttccaaacat tcacacgctt 6800
6801 tttgacatgt cggcggagga tttgatgtca atcatagcag aacacttcaa gcaaggcgac ccggtactgg agacggatat 6880
6881 cgcatcattc gacaaaagcc aagacgacgc tatggcgtta accggtctga tgatcttgga ggacctgggt gtggatcaac 6960
6961 cactactcga cttgatcgag tgcgcctttg gagaaatatc atccacccat ctacctacgg gtactcgttt taaattcggg 7040
7041 gcgatgatga aatccggaat gttcctcaca cttttgtca acacagtttt gaatgtcgtt atcgccagca gagtactaga 7120
7121 agagcggctt aaaacgtcca gatgtgcagc gttcattggc gacgacaaca tcatacatgg agtagtatct gacaaagaaa 7200
7201 tggctgagag gtgcgccacc tggctcaaca tggaggttaa gatcatcgac gcagtcatcg gtgagagacc acttacttc 7280
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7361 gggtaaaccg ctcccagccg acgacgagca agacgaagac agaagacgcg ctctgctaga tgaaacaaag gcgtggttta 7440
7441 gagtaggtat aacaggcact ttagcagtgg ccgtgacgac ccggtatgag gtagacaata ttacacctgt cctactggca 7520
7521 ttgagaactt ttgcccagag caaaagagca ttccaagcca tcagagggga aataaagcat ctctacggtg gtcctaaata 7600
7601 gtcagcatag tacatttcat ctgactaata ctacaacacc accacctcta gactcgagcg gccgccactg tgctggatat 7680
7681 ccaccacact ggactagtgg atccATGCAT GGAGATACAC CTACATTGCA TGAATATATG TTAGATTTGC 7760
7761 AACCAGAGAC AACTGATCTC TACTGTTATG AGCAATTAAA TGACAGCTCA GAGGAGGAGG ATGAAATAGA TGGTCCAGCT 7840
7841 GGACAAGCAG AACCGGACAG AGCCCATTAC AATATTGTAA CCTTTGTTG CAAGTGTGAC TCTACGCTTC GGTTGTGCGT 7920
7921 ACAAAGCACA CACGTAGACA TTCGTACTTT GGAAGACCTG TTAATGGGCA CACTAGGAAT TGTGTGCCCC ATCTGTTCTC 8000
8001 AAGGATCCAT GGCTCGTGCG GTCGGGATCG ACCTCGGGAC CACCAACTCC GTCGTCTCGG TTCTGGAAGG TGGCGACCCG 8080
8081 GTCGTCGTCG CCAACTCCGA GGGCTCCAGG ACCACCCCGT CAATTGTCGC GTTCGCCCGC AACGGTGAGG TGCTGGTCGG 8160
8161 CCAGCCCGCC AAGAACCAGG CAGTGACCAA CGTCGATCGC ACCGTGCGCT CGGTCAAGCG ACACATGGGC AGCGACTGGT 8240
8241 CCATAGAGAT TGACGGCAAG AAATACACCG CGCCGGAGAT CAGCGCCCGC ATTCTGATGA AGCTGAAGCG CGACGCCGAG 8320
8321 GCCTACCTCG GTGAGGACAT TACCGACGCG GTTATCACGA CGCCCGCCTA CTTCAATGAC GCCCAGCGTC AGGCCACCAA 8400
8401 GGACGCCGGC CAGATCGCCG GCCTCAACGT GCTGCGGATC GTCAACGAGC CGACCGCGGC CGCGCTGGCC TACGGCCTCG 8480
8481 ACAAGGGCGA GAAGGAGCAG CGAATCCTGG TCTTCGACTT GGGTGGTGGC ACTTTCGACG TTTCCCTGCT GGAGATCGGC 8560
8561 GAGGGTGTGG TTGAGGTCCG TGCCACTTCG GGTGACAACC ACCTCGGCGG CGACGACTGG GACCAGCGGG TCGTCGATTG 8640
8641 GCTGGTGGAC AAGTTCAAGG GCACCAGCGG CATCGATCTG ACCAAGGACA AGATGGCGAT GCAGCGGCTG CGGGAAGCCG 8720
8721 CCGAGAAGGC AAAGATCGAG CTGAGTTCGA GTCAGTCCAC CTCGATCAAC CTGCCCTACA TCACCGTCGA CGCCGACAAG 8800
8801 AACCCGTTGT TCTTAGACGA GCAGCTGACC CGCGCGGAGT TCCAACGGAT CACTCAGGAC CTGCTGGACC GCACTCGCAA 8880
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8881  GCCGTTCCAG TCGGTGATCG CTGACACCGG CATTTCGGTG TCGGAGATCG ATCACGTTGT GCTCGTGGGT GGTTCGACCC 8960
8961  GGATGCCCGC GGTGACCGAT CTGGTCAAGG AACTCACCGG CGGCAAGGAA CCCAACAAGG GCGTCAACCC CGATGAGGTT 9040
9041  GTCGCGGTGG GAGCCGCTCT GCAGGCCGGC GTCCTCAAGG GCGAGGTGAA AGACGTTCTG CTGCTTGATG TTACCCCGCT 9120
9121  GAGCCTGGGT ATCGAGACCA AGGGCGGGGT GATGACCAGG CTCATCGAGC GCAACACCAC GATCCCCACC AAGCGGTCGG 9200
9201  AGACTTTCAC CACCGCCGAC GACAACCAAC CGTCGGTGCA GATCCAGGTC TATCAGGGGG AGCGTGAGAT CGCCGCGCAC 9280
9281  AACAAGTTGC TCGGGTCCTT CGAGCTGACC GGCATCCCGC CGGCGCCGCG GGGGATTCCG CAGATCGAGG TCACTTTCGA 9360
9361  CATCGACGCC AACGGCATTG TGCACGTCAC CGCCAAGGAC AAGGGCACCG GCAAGGAGAA CACGATCCGA ATCCAGGAAG 9440
9441  GCTCGGGCCT GTCCAAGGAA GACATTGACC GCATGATCAA GGACGCCGAA GCGCACGCCG AGGAGGATCG CAAGCGTCGC 9520
9521  GAGGAGGCCG ATGTTCGTAA TCAAGCCGAG ACATTGGTCT ACCAGACGGA GAAGTTCGTC AAAGAACAGC GTGAGGCCGA 9600
9601  GGGTGGTTCG AAGGTACCTG AAGACACGCT GAACAAGGTT GATGCCGCGG TGGCGGAAGC GAAGGCGGCA CTTGGCGGAT 9680
9681  CGGATATTTC GGCCATCAAG TCGGCGATGG AGAAGCTGGG CCAGGAGTCG CAGGCTCTGG GGCAAGCGAT CTACGAAGCA 9760
9761  GCTCAGGCTG CGTCACAGGC CACTGGCGCT GCCCACCCCG GCTCGGCTGA TGAAAGCTTa agtttgtgag catgcaggcc 9840
9841  ttgggcccaa tgatccgacc agcaaaactc gatgtacttc cgaggaactg atgtgcataa tgcatcaggc tggtacatta 9920
9921  gatccccgct taccgcgggc aatatagcaa cactaaaaac tcgatgtact tccgaggaag cgcagtgcat aatgctgcgc 10000
10001 agtgttgcca cataaccact atattaacca tttatctagc ggacgccaaa aactcaatgt atttctgagg aagcgtggtg 10080
10081 cataatgcca cgcagcgtct gcataacttt tattatttct tttattaatc aacaaaattt tgttttaac atttcaaaaa 10160
10161 aaaaaaaaaa aaaaaaaaaa aagggaattc ctcgattaat taagcggccg ctcgagggga attaattctt 10240
10241 gaagacgaaa gggccaggtg gcacttttcg gggaaatgtg cgcggaaccc ctatttgttt atttttctaa atacattcaa 10320
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10481 agatgctgaa gatcagttgg gtgcacgagt gggttacatc gaactggatc tcaacagcgg taagatcctt gagagtttcc 10560
10561 gcccgaaga acgttttcca atgatgagca ctttaaagt tctgctatgt ggcgcggtat tatcccgtgt tgacgccggg 10640
10641 caagagcaac tcggtcgccg catacactat tctcagaatg acttggttga gtactcacca gtcacagaaa agcatcttac 10720
10721 ggatggcatg acagtaagag aattatgcag tgctgccata accatgagtg ataacactgc ggccaactta cttctgacaa 10800
10801 cgatcggagg accgaaggag ctaaccgctt ttttgcacaa catgggggat catgtaactc gccttgatcg ttgggaaccg 10880
10881 gagctgaatg aagccatacc aaacgacgag cgtgacacca cgatgcctgt agcaatggca acaacgttgc gcaaactatt 10960
10961 aactggcgaa ctacttactc tagcttcccg gcaacaatta atagactgga tggaggcgga taaagttgca ggaccacttc 11040
11041 tgcgctcggc ccttccggct ggctggttta ttgctgataa atctggagcc ggtgagcgtg ggtctcgcgg tatcattgca 11120
11121 gcactggggc cagatggtaa gccctcccgt atcgtagtta tctacacgac ggggagtcag gcaactatgg atgaacgaaa 11200
11201 tagacagatc gctgagatag gtgcctcact gattaagcat tggtaactgt cagaccaagt ttactcatat atactttaga 11280
11281 ttgatttaaa acttcatttt taatttaaaa ggatctaggt gaagatcctt tttgataatc tcatgaccaa aatcccttaa 11360
11361 cgtgagtttt cgttccactg agcgtcagac cccgtagaaa agatcaaagg atcttcttga gatcctttt ttctgcgcgt 11440
11441 aatctgctgc ttgcaaacaa aaaaccaccc gctaccagcg gtggtttgtt tgccggatca agagctacca actctttttc 11520
11521 cgaaggtaac tggcttcagc agagcgcaga taccaaatac tgtccttcta gtgtagccgt agttaggcca ccacttcaag 11600
11601 aactctgtag caccgcctac atacctcgct ctgctaatcc tgttaccagt ggctgctgcc agtggcgata agtcgtgtct 11680
11681 taccgggttg gactcaagac gatagttacc ggataaggcg cagcggtcgg gctgaacggg gggttcgtgc acacagccca 11760
11761 gcttgagcg aacgacctac accgaactga gatacctaca gcgtgagcat tgagaaagcg ccacgcttcc cgaagggaga 11840
11841 aaggcggaca ggtatccggt aagcggcagg gtcggaacag gagagcgcac gagggagctt ccagggggaa acgcctggta 11920
11921 tctttatagt cctgtcgggt ttcgccacct ctgacttgag cgtcgatttt tgtgatgctc gtcaggggggg cggagcctat 12000
12001 ggaaaaacgc cagcaacgcg agctcgtatg gacatattgt cgttagaacg cggctacaat taatacataa ccttatgtat 12080
12081 catacacata cgatttaggg gacactatag                                                      12110
          10        20        30        40        50        60        70        80
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pSCA1-E7-Hsp70 SEQ ID NO:20 The E7-Hsp70 fusion sequence is shown in bold, caps

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                |   10      |   20      |   30      |   40      |   50      |   60      |   70      |   80      |
      1 atggcggatg tgtgacatac acgacgccaa aagatttgt tccagctcct gccacctccg ctacgcgaga gattaaccac      80
     81 ccacgatggc cgccaaagtg catgttgata ttgaggctga cagccattc atcaagtctt tgcagaaggc atttccgtcg     160
    161 ttcgaggtgg agtcattgca ggtcacacca aatgaccatg caaatgccag agcatttcg cacctggcta ccaaattgat     240
    241 cgagcaggag actgacaaag acacactcat cttggatatc ggcagtgcgc cttccaggag aatgatgtct acgcacaaat     320
    321 accactgcgt atgccctatg cgcagcgcag aagaccccga aaggctcgat agctacgcaa agaaactggc agcggcctcc     400
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pcDNA3-FL-E7 SEQ ID NO:21 **FL- E7** fusion sequence is shown in bold, caps

	10	20	30	40	50	60	70	80	
1	gacggatcgg	gagatctccc	gatcccctat	ggtcgactct	cagtacaatc	tgctctgatg	ccgcatagtt	aagccagtat	80
81	ctgctccctg	cttgtgtgtt	ggaggtcgct	gagtagtgcg	cgagcaaaat	ttaagctaca	acaaggcaag	gcttgaccga	160
161	caattgcatg	aagaatctgc	ttagggttag	gcgttttgcg	ctgcttcgcg	atgtacgggc	cagatatacg	cgttgacatt	240
241	gattattgac	tagttattaa	tagtaatcaa	ttacggggtc	attagttcat	agcccatata	tggagttccg	cgttacataa	320
321	cttacggtaa	atggcccgcc	tggctgaccg	cccaacgacc	cccgcccatt	gacgtcaata	atgacgtatg	ttcccatagt	400
401	aacgccaata	gggactttcc	attgacgtca	atgggtggac	tatttacggt	aaactgccca	cttggcagta	catcaagtgt	480
481	atcatatgcc	aagtacgccc	cctattgacg	tcaatgacgg	taaatggccc	gcctggcatt	atgcccagta	catgacctta	560
561	tgggactttc	ctacttggca	gtacatctac	gtattagtca	tcgctattac	catggtgatg	cggttttggc	agtacatcaa	640
641	tgggcgtgga	tagcggtttg	actcacgggg	atttccaagt	ctccacccca	ttgacgtcaa	tgggagtttg	ttttggcacc	720
721	aaaatcaacg	ggactttcca	aaatgtcgta	acaactccgc	cccattgacg	caaatgggcg	gtaggcgtgt	acggtgggag	800
801	gtctatataa	gcagagctct	ctggctaact	agagaaccca	ctgcttactg	gcttatcgaa	attaatacga	ctcactatag	880
881	ggagacccaa	gctggctagc	gtttaaacgg	gccctctaga	**ATGACAGTGC**	**TGGCGCCAGC**	**CTGGAGCCCA**	**AATTCCTCCC**	**960**
961	**TGTTGCTGCT**	**GTTGCTGCTG**	**CTGAGTCCTT**	**GCCTGCGGGG**	**GACACCTGAC**	**TGTTACTTCA**	**GCCACAGTCC**	**CATCTCCTCC**	**1040**
1041	**AACTTCAAAG**	**TGAAGTTTAG**	**AGAGTTGACT**	**GACCACCTGC**	**TTAAAGATTA**	**CCCAGTCACT**	**GTGGCCGTCA**	**ATCTTCAGGA**	**1120**
1121	**CGAGAAGCAC**	**TGCAAGGCCT**	**TGTGGAGCCT**	**CTTCCTAGCC**	**CAGCGCTGGA**	**TAGAGCAACT**	**GAAGACTGTG**	**GCAGGGTCTA**	**1200**
1201	**AGATGCAAAC**	**GCTTCTGGAG**	**GACGTCAACA**	**CCGAGATACA**	**TTTTGTCACC**	**TCATGTACCT**	**TCCAGCCCCT**	**ACCAGAATGT**	**1280**
1281	**CTGCGATTCG**	**TCCAGACCAA**	**CATCTCCCAC**	**CTCCTGAAGG**	**ACACCTGCAC**	**ACAGCTGCTT**	**GCTCTGAAGC**	**CCTGTATCGG**	**1360**
1361	**GAAGGCCTGC**	**CAGAATTTCT**	**CTCGGTGCCT**	**GGAGGTGCAG**	**TGCCAGCCGG**	**ACTCCTCCAC**	**CCTGCTGCCC**	**CCAAGGAGTC**	**1440**
1441	**CCATAGCCCT**	**AGAAGCCACG**	**GAGCTCCCAG**	**AGCCTCGGCC**	**CAGGCAGGGA**	**TCCATGCATG**	**GAGATACACC**	**TACATTGCAT**	**1520**
1521	**GAATATATGT**	**TAGATTTGCA**	**ACCAGAGACA**	**ACTGATCTCT**	**ACTGTTATGA**	**GCAATTAAAT**	**GACAGCTCAG**	**AGGAGGAGGA**	**1600**
1601	**TGAAATAGAT**	**GGTCCAGCTG**	**GACAAGCAGA**	**ACCGGACAGA**	**GCCCATTACA**	**ATATTGTAAC**	**CTTTTGTTGC**	**AAGTGTGACT**	**1680**
1681	**CTACGCTTCG**	**GTTGTGCGTA**	**CAAAGCACAC**	**ACGTAGACAT**	**TCGTACTTTG**	**GAAGACCTGT**	**TAATGGGCAC**	**ACTAGGAATT**	**1760**
1761	GTGTGCCCCA	TCTGTTCTCA	GGATAAGCTT	aagtttaaac	cgctgatcag	cctcgactgt	gccttctagt	tgccagccat	1840
1841	ctgttgtttg	cccctccccc	gtgccttcct	tgaccctgga	aggtgccact	cccactgtcc	tttcctaata	aaatgaggaa	1920
1921	attgcatcgc	attgtctgag	taggtgtcat	tctattctgg	ggggtggggt	ggggcaggac	agcaaggggg	aggattggga	2000
2001	agacaatagc	aggcatgctg	gggatgcggt	gggctctatg	gcttctgagg	cggaaagaac	cagctggggc	tctaggggtt	2080
2081	atccccacgc	gccctgtagc	ggcgcattaa	gcgcggcggg	tgtggtggtt	acgcgcagcg	tgaccgctac	acttgccagc	2160
2161	gccctagcgc	ccgctccttt	cgctttcttc	ccttcctttc	tcgccacgtt	cgccggcttt	ccccgtcaag	ctctaaatcg	2240
2241	gggcatccct	ttagggttcc	gatttagtgc	tttacggcac	ctcgacccca	aaaaacttga	ttagggtgat	ggttcacgta	2320
2321	gtgggccatc	gccctgatag	acggtttttc	gccctttgac	gttggagtcc	acgttcttta	atagtggact	cttgttccaa	2400
2401	actggaacaa	cactcaaccc	tatctcggtc	tattcttttg	atttataagg	gattttgggg	atttcggcct	attggttaaa	2480
2481	aaatgagctg	atttaacaaa	aatttaacgc	gaattaattc	tgtggaatgt	gtgtcagtta	gggtgtggaa	agtccccagg	2560
2561	ctccccaggc	aggcagaagt	atgcaaagca	tgcatctcaa	ttagtcagca	accaggtgtg	gaaagtcccc	aggctcccca	2640
2641	gcaggcagaa	gtatgcaaag	catgcatctc	aattagtcag	caaccatagt	cccgccccta	actccgccca	tcccgccccт	2720
2721	aactccgccc	agttccgccc	attctccgcc	ccatggctga	ctaatttttt	ttatttatgc	agaggccgag	gccgcctctg	2800
2801	cctctgagct	attccagaag	tagtgaggag	gctttttgg	aggcctaggc	ttttgcaaaa	agctcccggg	agcttgtata	2880
2881	tccattttcg	gatctgatca	agagacagga	tgaggatcgt	ttcgcatgat	tgaacaagat	ggattgcacg	caggttctcc	2960
2961	ggccgcttgg	gtggagaggc	tattcggcta	tgactgggca	caacagacaa	tcggctgctc	tgatgccgcc	gtgttccggc	3040
3041	tgtcagcgca	ggggcgcccg	gttctttttg	tcaagaccga	cctgtccggt	gccctgaatg	aactgcagga	cgaggcagcg	3120

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3121  cggctatcgt ggctggccac gacgggcgtt ccttgcgcag ctgtgctcga cgttgtcact gaagcgggaa gggactggct  3200
3201  gctattgggc gaagtgccgg ggcaggatct cctgtcatct caccttgctc ctgccgagaa agtatccatc atggctgatg  3280
3281  caatgcggcg gctgcatacg cttgatccgg ctacctgccc attcgaccac caagcgaaac atcgcatcga gcgagcacgt  3360
3361  actcggatgg aagccggtct tgtcgatcag gatgatctgg acgaagagca tcaggggctc gcgccagccg aactgttcgc  3440
3441  caggctcaag gcgcgcatgc ccgacggcga ggatctcgtc gtgacccatg gcgatgcctg cttgccgaat atcatggtgg  3520
3521  aaaatggccg cttttctgga ttcatcgact gtggccggct gggtgtggcg gaccgctatc aggacatagc gttggctacc  3600
3601  cgtgatattg ctgaagagct tggcggcgaa tgggctgacc gcttcctcgt gctttacggt atcgccgctc ccgattcgca  3680
3681  gcgcatcgcc ttctatcgcc ttcttgacga gttcttctga gcgggactct ggggttcgaa atgaccgacc aagcgacgcc  3760
3761  caacctgcca tcacgagatt tcgattccac cgccgccttc tatgaaaggt tgggcttcgg aatcgttttc cgggacgccg  3840
3841  gctggatgat cctccagcgc ggggatctca tgctggagtt cttcgcccac cccaacttgt ttattgcagc ttataatggt  3920
3921  tacaaataaa gcaatagcat cacaaatttc acaaataaag catttttttc actgcattct agttgtggtt tgtccaaact  4000
4001  catcaatgta tcttatcatg tctgtatacc gtcgacctct agctagagct tggcgtaatc atggtcatag ctgtttcctg  4080
4081  tgtgaaattg ttatccgctc acaattccac acaacatacg agccggaagc ataaagtgta aagcctgggg tgcctaatga  4160
4161  gtgagctaac tcacattaat tgcgttgcgc tcactgcccg ctttccagtc gggaaacctg tcgtgccagc tgcattaatg  4240
4241  aatcggccaa cgcgcgggga gaggcggttt gcgtattggg cgctcttccg cttcctcgct cactgactcg ctgcgctcgg  4320
4321  tcgttcggct gcggcgagcg gtatcagctc actcaaaggc ggtaatacgg ttatccacag aatcagggga taacgcagga  4400
4401  aagaacatgt gagcaaaagg ccagcaaaag gccaggaacc gtaaaaaggc cgcgttgctg gcgtttttcc ataggctccg  4480
4481  ccccccctgac gagcatcaca aaaatcgacg ctcaagtcag aggtggcgaa acccgacagg actataaaga taccaggcgt  4560
4561  ttcccccctgg aagctccctc gtgcgctctc ctgttccgac cctgccgctt accggatacc tgtccgcctt tctcccttcg  4640
4641  ggaagcgtgg cgctttctca atgctcacgc tgtaggtatc tcagttcggt gtaggtcgtt cgctccaagc tgggctgtgt  4720
4721  gcacgaaccc cccgttcagc ccgaccgctg cgccttatcc ggtaactatc gtcttgagtc caacccggta agacacgact  4800
4801  tatcgccact ggcagcagcc actggtaaca ggattagcag agcgaggtat gtaggcggtg ctacagagtt cttgaagtgg  4880
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5041  aaaaaggatc tcaagaagat cctttgatct tttctacggg gtctgacgct cagtggaacg aaaactcacg ttaagggatt  5120
5121  ttggtcatga gattatcaaa aaggatcttc acctagatcc ttttaaatta aaaatgaagt tttaaatcaa tctaaagtat  5200
5201  atatgagtaa acttggtctg acagttacca atgcttaatc agtgaggcac ctatctcagc gatctgtcta tttcgttcat  5280
5281  ccatagttgc ctgactcccc gtcgtgtaga taactacgat acgggagggc ttaccatctg gccccagtgc tgcaatgata  5360
5361  ccgcgagacc cacgctcacc ggctccagat ttatcagcaa taaaccagcc agccggaagg gccgagcgca gaagtggtcc  5440
5441  tgcaacttta tccgcctcca tccagtctat taattgttgc cgggaagcta gagtaagtag ttcgccagtt aatagtttgc  5520
5521  gcaacgttgt tgccattgct acaggcatcg tggtgtcacg ctcgtcgttt ggtatggctt cattcagctc cggttcccaa  5600
5601  cgatcaaggc gagttacatg atcccccatg ttgtgcaaaa aagcggttag ctccttcggt cctccgatcg ttgtcagaag  5680
5681  taagttggcc gcagtgttat cactcatggt tatggcagca ctgcataatt ctcttactgt catgccatcc gtaagatgct  5760
5761  tttctgtgac tggtgagtac tcaaccaagt cattctgaga atagtgtatg cggcgaccga gttgctcttg cccggcgtca  5840
5841  atacgggata ataccgcgcc acatagcaga actttaaaag tgctcatcat tggaaaacgt tcttcggggc gaaaactctc  5920
5921  aaggatctta ccgctgttga gatccagttc gatgtaaccc actcgtgcac ccaactgatc ttcagcatct tttactttca  6000
6001  ccagcgtttc tgggtgagca aaaacaggaa ggcaaaatgc cgcaaaaaag ggaataaggg cgacacggaa atgttgaata  6080
6081  ctcatactct tccttttca atattattga agcatttatc agggttattg tctcatgagc ggatacatat ttgaatgtat  6160
6161  ttagaaaaat aaacaaatag gggttccgcg cacatttccc cgaaaagtgc cacctgacgt c                    6221
      |    10    |    20    |    30    |    40    |    50    |    60    |    70    |    80    |
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GENERAL RECOMBINANT DNA METHODS

Basic texts disclosing general methods of molecular biology, all of which are incorporated by reference, include: Sambrook, J *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989; Ausubel, FM *et al. Current Protocols in Molecular Biology*, Vol. 2, Wiley-Interscience, New York, (current edition); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); Glover, DM, ed, *DNA Cloning: A Practical Approach*, vol. I & II, IRL Press, 1985; Albers, B. *et al.*, *Molecular Biology of the Cell*, 2nd Ed., Garland Publishing, Inc., New York, NY (1989); Watson, JD *et al.*, *Recombinant DNA*, 2nd Ed., Scientific American Books, New York, 1992; and Old, RW *et al.*, *Principles of Gene Manipulation: An Introduction to Genetic Engineering*, 2nd Ed., University of California Press, Berkeley, CA (1981).

Unless otherwise indicated, a particular nucleic acid sequence is intended to encompasses conservative substitution variants thereof *(e.g.,* degenerate codon substitutions) and a complementary sequence. The term "nucleic acid" is synonymous with "polynucleotide" and is intended to include a gene, a cDNA molecule, an mRNA molecule, as well as a fragment of any of these such as an oligonucleotide, and further, equivalents thereof (explained more fully below). Sizes of nucleic acids are stated either as kilobases (kb) or base pairs (bp). These are estimates derived from agarose or polyacrylamide gel electrophoresis (PAGE), from nucleic acid sequences which are determined by the user or published. Protein size is stated as molecular mass in kilodaltons (kDa) or as length (number of amino acid residues). Protein size is estimated from PAGE, from sequencing, from presumptive amino acid sequences based on the coding nucleic acid sequence or from published amino acid sequences.

Specifically, cDNA molecules encoding the amino acid sequence corresponding to the fusion polypeptide of the present invention or fragments or derivatives thereof can be synthesized by the polymerase chain reaction (PCR) (see, for example, U.S. 4,683,202) using primers derived the sequence of the protein disclosed herein. These cDNA sequences can then be assembled into a eukaryotic or prokaryotic expression vector and the resulting vector can be used to direct the synthesis of the fusion polypeptide or its fragment or derivative by appropriate host cells, for example COS or CHO cells.

This invention includes isolated nucleic acids having a nucleotide sequence encoding the novel fusion polypeptides that comprise a MHC-I-PP or a DC-PP and an antigen, fragments thereof or equivalents thereof. The term nucleic acid as used herein is intended to include such fragments or equivalents. The nucleic acid sequences of this invention can be DNA or RNA.

A cDNA nucleotide sequence the fusion polypeptide can be obtained by isolating total mRNA from an appropriate cell line. Double stranded cDNA is prepared from total mRNA. cDNA can be inserted into a suitable plasmid, bacteriophage or viral vector using any one of a number of known techniques.

In reference to a nucleotide sequence, the term "equivalent" is intended to include sequences encoding structurally homologous and/or a functionally equivalent proteins. For example, a natural polymorphism Hsp70 or FL nucleotide sequence (especially at the third base of a codon) may be manifest as "silent" mutations which do not change the amino acid sequence. Furthermore, there may be one or more naturally occurring isoforms or related, immunologically cross-reactive family members of these proteins. Such isoforms or family members are defined as proteins that share function amino acid sequence similarity to, for example, Hsp70 or FL.

Fragment of Nucleic Acid

A fragment of the nucleic acid sequence is defined as a nucleotide sequence having fewer nucleotides than the nucleotide sequence encoding the full length MHC-I-PP or DC-PP protein, antigenic polypeptide or the fusion thereof.. This invention includes such nucleic acid fragments that encode polypeptides which retain (1) the ability of the fusion polypeptide to induce increases in frequency or reactivity of T cells, preferably CD8+ T cells, that are specific for the antigen part of the fusion polypeptide.

For example, a nucleic acid fragment as intended herein encodes a FL polypeptide that retains the ability to improve the immunogenicity of an antigen when administered as a fusion polypeptide with an antigenic polypeptide or peptide.

Generally, the nucleic acid sequence encoding a fragment of a FL or Hsp70 polypeptide comprises of nucleotides from the sequence encoding the mature protein (or an active fragment thereof such as the FL ECD or the C-terminal domain of Hsp70.

Nucleic acid sequences of this invention may also include linker sequences, natural or modified restriction endonuclease sites and other sequences that are useful for manipulations related to cloning, expression or purification of encoded protein or fragments. These and other modifications of nucleic acid sequences are described herein or are well-known in the art.

The techniques for assembling and expressing DNA coding sequences for MHC-I-PP or DC-pp types of proteins, and DNA coding sequences for antigenic polypeptides, include synthesis of oligonucleotides, PCR, transforming cells, constructing vectors, expression systems, and the like; these are well-established in the art such that those of ordinary skill are familiar with standard resource materials, specific conditions and procedures.

EXPRESSION VECTORS AND HOST CELLS

This invention includes an expression vector comprising a nucleic acid sequence encoding a MHC-I-PP/antigen fusion polypeptide or a DC-PP/antigen fusion polypeptide operably linked to at least one regulatory sequence.

The term "expression vector" or "expression cassette" as used herein refers to a nucleotide sequence which is capable of affecting expression of a protein coding sequence in a host compatible with such sequences. Expression cassettes include at least a promoter operably linked with the polypeptide coding sequence; and, optionally, with other sequences, *e.g.*, transcription termination signals. Additional factors necessary or helpful in effecting expression may also be included, *e.g.*, enhancers.

"Operably linked" means that the coding sequence is linked to a regulatory sequence in a manner that allows expression of the coding sequence. Known regulatory sequences are selected to direct expression of the desired protein in an appropriate host cell. Accordingly, the term "regulatory sequence" includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in, for example, Goeddel, *Gene Expression Technology. Methods in Enzymology,* vol. 185, Academic Press, San Diego, Calif. (1990)).

Thus, expression cassettes include plasmids, recombinant viruses, any form of a recombinant "naked DNA" vector, and the like. A "vector" comprises a nucleic acid which can infect, transfect, transiently or permanently transduce a cell. It will be recognized that a vector can be a naked nucleic acid, or a nucleic acid complexed with protein or lipid. The vector optionally comprises viral or bacterial nucleic acids and/or proteins, and/or membranes (*e.g.*, a cell membrane, a viral lipid envelope, *etc.*). Vectors include, but are not limited to replicons (*e.g.*, RNA replicons (see Example 1, below), bacteriophages) to which fragments of DNA may be attached and become replicated. Vectors thus include, but are not limited to RNA, autonomous self-replicating circular or linear DNA or RNA, *e.g.*, plasmids, viruses, and the like (U.S. Patent No. 5,217,879), and includes both the expression and nonexpression plasmids. Where a recombinant microorganism or cell culture is described as hosting an "expression vector" this includes both extrachromosomal circular and linear DNA and DNA that has been incorporated into the host chromosome(s). Where a vector is being maintained by a host cell, the vector may either be stably replicated by the cells during mitosis as an autonomous structure, or is incorporated within the host's genome.

Those skilled in the art appreciate that the particular design of an expression vector of this invention depends on considerations such as the host cell to be transfected and/or the type of protein to be expressed.

The present expression vectors comprise the full range of nucleic acid molecules encoding the various embodiments of the fusion polypeptide and its functional derivatives (defined herein) including polypeptide fragments, variants, *etc.*

Such expression vectors are used to transfect host cells (*in vitro*, *ex vivo* or *in vivo*) for expression of the DNA and production of the encoded proteins which include fusion proteins or peptides. It will be understood that a genetically modified cell expressing the fusion polypeptide may transiently express the exogenous DNA for a time sufficient for the cell to be useful for its stated purpose.

The present in invention provides methods for producing the fusion polypeptides, fragments and derivatives. For example, a host cell transfected with a nucleic acid vector that encodes the fusion polypeptide is cultured under appropriate conditions to allow expression of the polypeptide.

Host cells may also be transfected with one or more expression vectors that singly or in combination comprise DNA encoding at least a portion of the fusion polypeptide and DNA encoding at least a portion of a second protein, so that the host cells produce yet further fusion polypeptides that include both the portions.

A culture typically includes host cells, appropriate growth media and other byproducts. Suitable culture media are well known in the art. The fusion polypeptide can be isolated from medium or cell lysates using conventional techniques for purifying proteins and peptides, including ammonium sulfate precipitation, fractionation column chromatography (e.g. ion exchange, gel filtration, affinity chromatography, etc.) and/or electrophoresis (see generally, "Enzyme Purification and Related Techniques", *Methods in Enzymology,* 22:233-577 (1971)). Once purified, partially or to homogeneity, the recombinant polypeptides of the invention can be utilized in pharmaceutical compositions as described in more detail herein.

The term "isolated" as used herein, when referring to a molecule or composition, such as a MHC-I-PP or nucleic acid coding therefor, means that the molecule or composition is separated from at least one other compound (protein, other nucleic acid, *etc.*) or from other contaminants with which it is natively associated or becomes associated during processing.. An isolated composition can also be substantially pure. An isolated composition can be in a homogeneous state and can be dry or in aqueous solution. Purity and homogeneity can be determined, for example, using analytical chemical techniques such as polyacrylamide gel electrophoresis (PAGE) or high performance liquid chromatography (HPLC). Even where a protein has been isolated so as to appear as a homogenous or dominant band in a gel pattern, there are trace contaminants which co-purify with it.

Prokaryotic or eukaryotic host cells transformed or transfected to express the fusion polypeptide or a homologue or functional derivative thereof are within the scope of the invention. For example, the fusion polypeptide may be expressed in bacterial cells such as *E. coli*, insect cells (baculovirus), yeast, or mammalian cells such as Chinese hamster ovary cells (CHO) or human cells. Other suitable host cells may be found in Goeddel, (1990) *supra* or are otherwise known to those skilled in the art.

Expression in eukaryotic cells leads to partial or complete glycosylation and/or formation of relevant inter- or intra-chain disulfide bonds of the recombinant protein.

Although preferred vectors are described in the Examples, other examples of expression vectors are provided here. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari *et al.*, (1987) *EMBO J.* 6:229-234), pMFa (Kurjan *et al.* (1982) *Cell* 30:933-943), pJRY88 (Schultz *et al.*, (1987) *Gene* 54:113-123), and pYES2 (Invitrogen Corporation, San Diego, Calif.). Baculovirus vectors available for expression of proteins in cultured insect cells (SF 9 cells) include the pAc series (Smith *et al.*, (1983) *Mol. Cell Biol.* 3:2156-2165,) and the pVL series (Lucklow, V. A., and Summers, M. D., (1989) *Virology* 170:31-39). Generally, COS cells (Gluzman, Y., (1981) *Cell* 23:175-182) are used in conjunction with such vectors as pCDM 8 (Aruffo A. and Seed, B., *supra,* for transient amplification/expression in mammalian cells, while CHO (*dhfr*-negative CHO) cells are used with vectors such as pMT2PC (Kaufman *et al.* (1987), *EMBO J.* 6:187-195) for stable amplification/expression in mammalian cells. The NS0 myeloma cell line (a glutamine synthetase expression system.) is available from Celltech Ltd.

Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the reporter group and the target protein to enable separation of the target protein from the reporter group subsequent to purification of the fusion protein. Proteolytic enzymes for such cleavage and their recognition sequences include Factor Xa, thrombin and enterokinase.

Typical fusion expression vectors include pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase, maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Inducible non-fusion expression vectors include pTrc (Amann *et al.*, (1988) *Gene* 69:301-315) and pET 11d (Studier *et al.*, *Gene Expression Technology:Methods in Enzymology* 185, Academic Press, San Diego, Calif. (1990) 60-89). While target gene expression relies on host RNA polymerase transcription from the hybrid trp-lac fusion promoter in pTrc, expression of target genes inserted into pET 11d relies on transcription from the T7 gn10-lacO fusion promoter mediated by coexpressed viral RNA polymerase (T7gn1). Th is viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident λ prophage harboring a T7gn1 under the transcriptional control of the lacUV 5 promoter.

One embodiment of this invention is a transfected cell which expresses novel fusion polypeptide.

Vector Construction

Construction of suitable vectors containing the desired coding and control sequences employs standard ligation and restriction techniques which are well understood in the art. Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and re-ligated in the form desired.

The DNA sequences which form the vectors are available from a number of sources. Backbone vectors and control systems are generally found on available "host" vectors which are used for the bulk of the sequences in construction. For the pertinent coding sequence, initial construction may be, and usually is, a matter of retrieving the appropriate sequences from cDNA or genomic DNA libraries. However, once the sequence is disclosed it is possible to synthesize the entire gene sequence *in vitro* starting from the individual nucleotide derivatives. The entire gene sequence for genes of sizeable length, *e.g.*, 500-1000 bp may be prepared by synthesizing individual overlapping complementary oligonucleotides and filling in single stranded nonoverlapping portions using DNA polymerase in the presence of the deoxyribonucleotide triphosphates. This approach has been used successfully in the construction of several genes of known sequence. See, for example, Edge, M. D., *Nature* (1981) 292:756; Nambair, K. P., *et al.*, *Science* (1984) 223:1299; and Jay, E., *J Biol Chem* (1984) 259:6311.

Synthetic oligonucleotides are prepared by either the phosphotriester method as described by references cited above or the phosphoramidite method as described by Beaucage, S. L., and Caruthers, M. H., *Tet Lett* (1981) 22:1859; and Matteucci, M. D., and Caruthers, M. H., *J Am Chem Soc* (1981) 103:3185 and can be prepared using commercially available automated oligonucleotide synthesizers. Kinase treatment of single strands prior to annealing or for labeling is achieved using an excess, e.g., about 10 units of polynucleotide kinase to 1 nmole substrate in the presence of 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1-2 mM ATP, 1.7 pmoles γ-^{32}P-ATP (2.9 mCi/mmole), 0.1 mM spermidine, 0.1 mM EDTA.

Once the components of the desired vectors are thus available, they can be excised and ligated using standard restriction and ligation procedures. Site-specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, *e.g.*, New England Biolabs, Product Catalog. In general, about 1 mg of plasmid or DNA sequence is cleaved by one unit of enzyme in about 20 ml of buffer solution; in the examples herein, typically, an excess of restriction enzyme is used to insure complete digestion of the DNA substrate. Incubation times of about one hour to two hours at about 37°C. are workable, although variations can be tolerated. After each incubation, protein is removed by extraction with phenol/chloroform, and may be followed by ether extraction, and the nucleic acid recovered from aqueous fractions by precipitation with ethanol. If desired, size separation of the cleaved fragments may

be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in *Methods in Enzymology* (1980) 65:499-560.

Restriction cleaved fragments may be blunt ended by treating with the large fragment of *E. coli* DNA polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTPs) using incubation times of about 15 to 25 min at 20° to 25° C. in 50 mM Tris pH 7.6, 50 mM NaCl, 6 mM $MgCl_2$, 6 mM DTT and 0.1-1.0 mM dNTPs. The Klenow fragment fills in at 5′ single-stranded overhangs but chews back protruding 3′ single strands, even though the four dNTPs are present. If desired, selective repair can be performed by supplying only one of the, or selected, dNTPs within the limitations dictated by the nature of the overhang. After treatment with Klenow, the mixture is extracted with phenol/chloroform and ethanol precipitated. Treatment under appropriate conditions with S1 nuclease or BAL-31 results in hydrolysis of any single-stranded portion.

Ligations are typically performed in 15-50 ml volumes under the following standard conditions and temperatures: for example, 20 mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 10 mM DTT, 33 μg/ml BSA, 10-50mM NaCl, and either 40 μM ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0° C. (for "sticky end" ligation) or 1mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14° C. (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 33-100 μg/ml total DNA concentrations (5-100 nM total end concentration). Intermolecular blunt end ligations are performed at 1 mM total ends concentration.

In vector construction employing "vector fragments", the fragment is commonly treated with bacterial alkaline phosphatase (BAP) or calf intestinal alkaline phosphatase (CIAP) in order to remove the 5' phosphate and prevent self-ligation. Digestions are conducted at pH 8 in approximately 10 mM Tris-HCl, 1 mM EDTA using BAP or CIAP at about 1 unit/mg vector at 60° for about one hour. The preparation is extracted with phenol/chloroform and ethanol precipitated. Alternatively, re-ligation can be prevented in vectors which have been double digested by additional restriction enzyme and separation of the unwanted fragments.

Any of a number of methods are used to introduce mutations into the coding sequence to generate the variants of the invention. These mutations include simple deletions or insertions, systematic deletions, insertions or substitutions of clusters of bases or substitutions of single bases.

For example, modifications of the MHC-I-PP or DC-pp or the antigenic polypeptide DNA sequence are created by site-directed mutagenesis, a well-known technique for which protocols and reagents are commercially available (Zoller, MJ *et al., Nucleic Acids Res* (1982) 10:6487-6500 and Adelman, JP *et al., DNA* (1983) 2:183-193)). Correct ligations for plasmid construction are confirmed, for example, by first transforming *E. coli* strain MC1061 (Casadaban, M., *et al., J Mol Biol* (1980) 138:179-207) or other suitable host with the ligation mixture. Using conventional methods,

transformants are selected based on the presence of the ampicillin-, tetracycline- or other antibiotic resistance gene (or other selectable marker) depending on the mode of plasmid construction. Plasmids are then prepared from the transformants with optional chloramphenicol amplification optionally following chloramphenicol amplification ((Clewell, DB *et al.* , *Proc Natl Acad Sci USA* (1969) 62:1159; Clewell, D. B., *J Bacteriol* (1972) 110:667). Several mini DNA preps are commonly used. See, *e.g.*,, Holmes, DS, *et al.*, *Anal Biochem* (1981) 114:193-197; Birnboim, HC *et al.* , *Nucleic Acids Res* (1979) 7:1513-1523. The isolated DNA is analyzed by restriction and/or sequenced by the dideoxy nucleotide method of Sanger (*Proc Natl Acad Sci USA* (1977) 74:5463) as further described by Messing, *et al.*, *Nucleic Acids Res* (1981) 9:309, or by the method of Maxam *et al. Methods in Enzymology* (1980) 65:499.

Vector DNA can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming host cells can be found in Sambrook *et al. supra* and other standard texts.

Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the reporter group and the target protein to enable separation of the target protein from the reporter group subsequent to purification of the fusion protein. Proteolytic enzymes for such cleavage and their recognition sequences include Factor Xa, thrombin and enterokinase.

Known fusion expression vectors include pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase, maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Inducible non-fusion expression vectors include pTrc (Amann *et al.*, (1988) *Gene* 69:301-315) and pET 11d (Studier *et al.*, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, Calif. (1990) 60-89). While target gene expression relies on host RNA polymerase transcription from the hybrid trp-lac fusion promoter in pTrc, expression of target genes inserted into pET 11d relies on transcription from the T7 gn10-lacO fusion promoter mediated by coexpressed viral RNA polymerase (T7gn1). Th is viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident λ prophage harboring a T7gn1 under the transcriptional control of the lacUV 5 promoter.

Promoters and Enhancers

A promoter region of a DNA or RNA molecule binds RNA polymerase and promotes the transcription of an "operably linked" nucleic acid sequence. As used herein, a "promoter sequence" is the nucleotide sequence of the promoter which is found on that strand of the DNA or RNA which is

transcribed by the RNA polymerase. Two sequences of a nucleic acid molecule, such as a promoter and a coding sequence, are "operably linked" when they are linked to each other in a manner which permits both sequences to be transcribed onto the same RNA transcript or permits an RNA transcript begun in one sequence to be extended into the second sequence. Thus, two sequences, such as a promoter sequence and a coding sequence of DNA or RNA are operably linked if transcription commencing in the promoter sequence will produce an RNA transcript of the operably linked coding sequence. In order to be "operably linked" it is not necessary that two sequences be immediately adjacent to one another in the linear sequence.

The preferred promoter sequences of the present invention must be operable in mammalian cells and may be either eukaryotic or viral promoters. Although preferred promoters are described in the Examples, other useful promoters and regulatory elements are discussed below. Suitable promoters may be inducible, repressible or constitutive. A "constitutive" promoter is one which is active under most conditions encountered in the cell's environmental and throughout development. An "inducible" promoter is one which is under environmental or developmental regulation. A "tissue specific" promoter is active in certain tissue types of an organism. An example of a constitutive promoter is the viral promoter MSV-LTR, which is efficient and active in a variety of cell types, and, in contrast to most other promoters, has the same enhancing activity in arrested and growing cells. Other preferred viral promoters include that present in the CMV-LTR (from cytomegalovirus) (Bashart, M. *et al.*, *Cell 41*:521 (1985)) or in the RSV-LTR (from Rous sarcoma virus) (Gorman, C.M., *Proc. Natl. Acad. Sci. USA 79*:6777 (1982). Also useful are the promoter of the mouse metallothionein I gene (Hamer, D., *et al.*, *J. Mol. Appl. Gen. 1*:273-288 (1982)); the TK promoter of Herpes virus (McKnight, S., *Cell 31*:355-365 (1982)); the SV40 early promoter (Benoist, C., *et al.*, *Nature 290*:304-310 (1981)); and the yeast *gal4* gene promoter (Johnston, S.A., *et al.*, *Proc. Natl. Acad. Sci. (USA) 79*:6971-6975 (1982); Silver, P.A., *et al.*, *Proc. Natl. Acad. Sci. (USA) 81*:5951-5955 (1984)). Other illustrative descriptions of transcriptional factor association with promoter regions and the separate activation and DNA binding of transcription factors include: Keegan *et al.*, *Nature* (1986) *231*:699; Fields *et al.*, *Nature* (1989) 340:245; Jones, *Cell* (1990) *61*:9; Lewin, Cell (1990) *61*:1161; Ptashne *et al.*, *Nature* (1990) *346*:329; Adams *et al.*, *Cell* (1993) 72:306. The relevant disclosure of all of these above-listed references is hereby incorporated by reference.

The promoter region may further include an octamer region which may also function as a tissue specific enhancer, by interacting with certain proteins found in the specific tissue. The enhancer domain of the DNA construct of the present invention is one which is specific for the target cells to be transfected, or is highly activated by cellular factors of such target cells. Examples of vectors (plasmid or retrovirus) are disclosed in (Roy-Burman *et al.*, U.S. Patent No. 5,112,767). For a general discussion

of enhancers and their actions in transcription, see, Lewin, B.M., *Genes IV*, Oxford University Press, Oxford, (1990), pp. 552-576. Particularly useful are retroviral enhancers (*e.g.*, viral LTR). The enhancer is preferably placed upstream from the promoter with which it interacts to stimulate gene expression. For use with retroviral vectors, the endogenous viral LTR may be rendered enhancer-less and substituted with other desired enhancer sequences which confer tissue specificity or other desirable properties such as transcriptional efficiency.

The nucleic acid sequences of the invention can also be chemically synthesized using standard techniques. Various methods of chemically synthesizing polydeoxynucleotides are known, including solid-phase synthesis which, like peptide synthesis, has been fully automated with commercially available DNA synthesizers (See, *e.g.*, Itakura *et al.* U.S. Pat. No. 4,598,049; Caruthers *et al.* U.S. Pat. No. 4,458,066; and Itakura U.S. Pat. Nos. 4,401,796 and 4,373,071, incorporated by reference herein).

PROTEINS AND POLYPEPTIDES

The terms "polypeptide," "protein," and "peptide" when reeferring to compositions of the invention are meant to include variants, analogues, and mimetics with structures and/or activity that substantially correspond to the polypeptide or peptide from which the variant,e tc. was derived.

The present invention includes an "isolated" fusion polypeptide comprising a MHC-I-PP and/or a DC-PP linked to an antigenic polypeptide. A preferred MHC-I-PP is Hsp70. A preferred DC-PP if FL or the ECD or FL. Preferred fusion polyhpeptides are Hsp70/E7 (SEQ ID NO:8) and FL-E7 (SEQ ID NO:12). While the present disclosure exemplifies the full length Hsp70 and the ECD of FL, it is to be understood that homologues of Hsp70 from other bacteria or from eukaryotic origin, homlogues of FL or its ECD,, and mutants thereof that possess the characteristics disclosed herein are intended within the scope of this invention.

The term "chimeric" or "fusion" polypeptide or protein refers to a composition comprising at least one polypeptide or peptide sequence or domain that is chemically bound in a linear fashion with a second polypeptide or peptide domain. One embodiment of this invention is an isolated or recombinant nucleic acid molecule encoding a fusion protein comprising at least two domains, wherein the first domain comprises an MHC-I-PP or a DC-PP *e.g.*, Hsp60 or FL, and the second domain comprising an antigenic epitope, *e.g.*, an MHC class I-binding peptide epitope. Additional domains can comprise a polypeptide, peptide, polysaccharide, or the like. The "fusion" can be an association generated by a peptide bond, a chemical linking, a charge interaction (*e.g.*, electrostatic attractions, such as salt bridges, H-bonding, *etc.*) or the like. If the polypeptides are recombinant, the "fusion protein" can be translated from a common mRNA. Alternatively, the compositions of the domains can be linked by any chemical or electrostatic means. The chimeric molecules of the invention (*e.g.*, MHC-I-PP- fusion proteins) can also include additional sequences, *e.g.*, linkers, epitope tags, enzyme cleavage recognition sequences,

signal sequences, secretion signals, and the like. Alternatively, a peptide can be linked to a carrier simply to facilitate manipulation or identification/ location of the peptide.

Also included is a "functional derivative" of Hsp70 or FL, which refers to an amino acid substitution variant, a "fragment," or a "chemical derivative" of the protein, which terms are defined below. A functional derivative retains measurable (a) Hsp70-like or (b) FL-like activity, preferably that of promoting immunogenicity of one or more antigenic epitopes fused thereto by either (a) promoting presentation by class I pathways or (b) promoting maturation or activation of APCs, which permits the "functional derivative's" utility in accordance with the present invention. "Functional derivatives" encompass "variants" and "fragments" regardless of whether the terms are used in the conjunctive or the alternative herein.

A functional homologue must possess the above biochemical and biological activity. In view of this functional characterization, use of homologous Hsp70 or FL proteins including proteins not yet discovered, fall within the scope of the invention if these proteins have sequence similarity and the recited biochemical and biological activity.

To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred method of alignment, Cys residues are aligned.

In a preferred embodiment, the length of a sequence being compared is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence (*e.g.*, Hsp70, SEQ ID NO:4 and FL-ECD, SEQ ID NO:10). The amino acid residues (or nucleotides) at corresponding amino acid (or nucleotide) positions are then compared. When a position in the first sequence is occupied by the same amino acid residue (or nucleotide) as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (*J. Mol. Biol.*

48:444-453 (1970) algorithm which has been incorporated into the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Meyers and W. Miller (CABIOS, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases, for example, to identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul *et al.* (1990) *J. Mol. Biol. 215*:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to Hsp70 or FL nucleic acid molecules. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to HVP22 protein molecules. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res. 25*:3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

Thus, a homologue of Hsp70 or of FL described above is characterized as having (a) functional activity of native Hsp70 or FL and (b) sequence similarity to a native Hsp70 protein (such as SEQ ID NO:4) or native FL (SEQ ID NO:10) when determined as above, of at least about 20% (at the amino acid level), preferably at least about 40%, more preferably at least about 70%, even more preferably at least about 90%.

It is within the skill in the art to obtain and express such a protein using DNA probes based on the disclosed sequences of Hsp70 or FL. Then, the fusion protein's biochemical and biological activity can be tested readily using art-recognized methods such as those described herein, for example, a T cell proliferation, cytokine secretion or a cytolytic assay, or an *in vivo* assay of tumor protection or tumor therapy. A biological assay of the stimulation of antigen-specific T cell reactivity will indicate whether the homologue has the requisite activity to qualify as a "functional" homologue.

A "variant" refers to a molecule substantially identical to either the full protein or to a fragment thereof in which one or more amino acid residues have been replaced (substitution variant) or which has

one or several residues deleted (deletion variant) or added (addition variant). A "fragment" of Hsp70 or FL refers to any subset of the molecule, that is, a shorter polypeptide of the full-length protein.

A number of processes can be used to generate fragments, mutants and variants of the isolated DNA sequence. Small subregions or fragments of the nucleic acid encoding the spreading protein, for example 1-30 bases in length, can be prepared by standard, chemical synthesis. Antisense oligonucleotides and primers for use in the generation of larger synthetic fragment.

A preferred group of variants are those in which at least one amino acid residue and preferably, only one, has been substituted by different residue. For a detailed description of protein chemistry and structure, see Schulz, GE *et al.*, *Principles of Protein Structure*, Springer-Verlag, New York, 1978, and Creighton, T.E., *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, 1983, which are hereby incorporated by reference. The types of substitutions that may be made in the protein molecule may be based on analysis of the frequencies of amino acid changes between a homologous protein of different species, such as those presented in Table 1-2 of Schulz *et al. (supra)* and Figure 3-9 of Creighton (*supra*). Based on such an analysis, conservative substitutions are defined herein as exchanges within one of the following five groups:

1	Small aliphatic, nonpolar or slightly polar residues	Ala, Ser, Thr (Pro, Gly);
2	Polar, negatively charged residues and their amides	Asp, Asn, Glu, Gln;
3	Polar, positively charged residues	His, Arg, Lys;
4	Large aliphatic, nonpolar residues	Met, Leu, Ile, Val (Cys)
5	Large aromatic residues	Phe, Tyr, Trp.

The three amino acid residues in parentheses above have special roles in protein architecture. Gly is the only residue lacking a side chain and thus imparts flexibility to the chain. Pro, because of its unusual geometry, tightly constrains the chain. Cys can participate in disulfide bond formation, which is important in protein folding.

More substantial changes in biochemical, functional (or immunological) properties are made by selecting substitutions that are less conservative, such as between, rather than within, the above five groups. Such changes will differ more significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Examples of such substitutions are (i) substitution of Gly and/or Pro by another amino acid or deletion or insertion of Gly or Pro; (ii) substitution of a hydrophilic residue, *e.g.*, Ser or Thr, for (or by) a hydrophobic residue, *e.g.*,, Leu, Ile, Phe, Val or Ala; (iii) substitution of a Cys residue for (or by) any other residue; (iv) substitution of a residue having an electropositive side chain, *e.g.*,, Lys, Arg or His, for (or by) a

residue having an electronegative charge, *e.g.*,, Glu or Asp; or (v) substitution of a residue having a bulky side chain, *e.g.*, Phe, for (or by) a residue not having such a side chain, *e.g.*, Gly.

Most acceptable deletions, insertions and substitutions according to the present invention are those that do not produce radical changes in the characteristics of the wild-type or native protein in terms of its intercellular spreading activity and its ability to stimulate antigen specific T cell reactivity to an antigenic epitope or epitopes that are fused to the spreading protein. However, when it is difficult to predict the exact effect of the substitution, deletion or insertion in advance of doing so, one skilled in the art will appreciate that the effect can be evaluated by routine screening assays such as those described here, without requiring undue experimentation.

Whereas shorter chain variants can be made by chemical synthesis, for the present invention, the preferred longer chain variants are typically made by site-specific mutagenesis of the nucleic acid encoding the polypeptide, expression of the variant nucleic acid in cell culture, and, optionally, purification of the polypeptide from the cell culture, for example, by immunoaffinity chromatography using specific antibody immobilized to a column (to absorb the variant by binding to at least one epitope).

The term "chemically linked" refers to any chemical bonding of two moieties, *e.g.*, as in one embodiment of the invention, where an **MHC-I-PP** or DC-PP is chemically linked to an antigenic peptide. Such chemical linking includes the peptide bonds of a recombinantly or *in vivo* generated fusion protein.

Chemical Derivatives

"Chemical derivatives" of the polypeptide or fusion polypeptide of the invention contain additional chemical moieties not normally a part of the protein. Covalent modifications of the polypeptide are included within the scope of this invention. Such derivatized moieties may improve the solubility, absorption, biological half life, and the like. Moieties capable of mediating such effects are disclosed, for example, in *Remington's Pharmaceutical Sciences*, 16th ed., Mack Publishing Co., Easton, PA (1980).

Such modifications may be introduced into the molecule by reacting targeted amino acid residues of the polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues. Another modification is cyclization of the protein. Examples of chemical derivatives of the polypeptide follow.

Lysinyl and amino terminal residues are derivatized with succinic or other carboxylic acid anhydrides. Derivatization with a cyclic carboxylic anhydride has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride;

trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Carboxyl side groups, aspartyl or glutamyl, may be selectively modified by reaction with carbodiimides (R-N=C=N-R') such as 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues can be converted to asparaginyl and glutaminyl residues by reaction with ammonia.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the amino group of lysine (Creighton, *supra,* pp. 79-86), acetylation of the N-terminal amine, and amidation of the C-terminal carboxyl groups.

Also included are peptides wherein one or more D-amino acids are substituted for one or more L-amino acids.

Multimeric Peptides

The present invention also includes longer polypeptides in which a basic peptidic sequence obtained from the sequence of either the MHC-I-PP or the DC-PP, or the antigenic polypeptide or peptide unit, is repeated from about two to about 100 times, with or without intervening spacers or linkers. It is understood that such multimers may be built from any of the peptide variants defined herein. Moreover, a peptide multimer may comprise different combinations of peptide monomers and the disclosed substitution variants thereof. Such oligomeric or multimeric peptides can be made by chemical synthesis or by recombinant DNA techniques as discussed herein. When produced chemically, the oligomers preferably have from 2-12repeats of the basic peptide sequence. When produced recombinantly, the multimers may have as many repeats as the expression system permits, for example from two to about 100 repeats.

In tandem multimers, preferably dimers and trimers, of the fusion polypeptide, the chains bonded by interchain disulfide bonds or other "artificial" covalent bonds between the chains such that the chains are "side-by-side" rather than "end to end."

THERAPEUTIC COMPOSITIONS AND THEIR ADMINISTRATION

A vaccine composition comprising the nucleic acid encoding the fusion polypeptide, or a cell expressing this nucleic acid is administered to a mammalian subject, preferably a human. The vaccine composition is administered in a pharmaceutically acceptable carrier in a biologically effective or a therapeutically effective amount . The composition may be given alone or in combination with another protein or peptide such as an immunostimulatory molecule. Treatment may include administration of an adjuvant, used in its broadest sense to include any nonspecific immune stimulating compound such as

an interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether.

A therapeutically effective amount is a dosage that, when given for an effective period of time, achieves the desired immunological or clinical effect.

A therapeutically active amount of a nucleic acid encoding the fusion polypeptide may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the peptide to elicit a desired response in the individual. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation. A therapeutically effective amounts of the protein, in cell associated form may be stated in terms of the protein or cell equivalents.

Thus an effective amount is between about 1 nanogram and about 10 milligram per kilogram of body weight of the recipient, more preferably between about 0.1 μg and 1 μg/kg. Dosage forms suitable for internal administration preferably contain (for the latter dose range) from about 0.01 μg to 100 μg of active ingredient per unit. The active ingredient may vary from 0.5 to 95% by weight based on the total weight of the composition. Alternatively, an effective dose of cells expressing the nucleic acid is between about 10^4 and 10^8 cells. Those skilled in the art of immunotherapy will be able to adjust these doses without undue experimentation.

The active compound may be administered in a convenient manner, *e.g.*, injection by a convenient and effective route. Preferred routes include subcutaneous, intradermal, intravenous and intramuscular routes. Other possible routes include oral administration, intrathecal, inhalation, transdermal application, or rectal administration. For the treatment of existing tumors which have not been completely resected or which have recurred, direct intratumoral injection is also intended.

Depending on the route of administration, the active compound may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound. Thus it may be necessary to coat the composition with, or co-administer the composition with, a material to prevent its inactivation. For example, an enzyme inhibitors of nucleases or proteases (*e.g.*, pancreatic trypsin inhibitor, diisopropylfluorophosphate and trasylol).or in an appropriate carrier such as liposomes (including water-in-oil-in-water emulsions as well as conventional liposomes (Strejan *et al.*, (1984) *J. Neuroimmunol* 7:27).

As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof

in the therapeutic compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Preferred pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Pharmaceutical compositions suitable for injection include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. Isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride may be included in the pharmaceutical composition. In all cases, the composition should be sterile and should be fluid. It should be stable under the conditions of manufacture and storage and must include preservatives that prevent contamination with microorganisms such as bacteria and fungi. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants.

Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like.

Compositions are preferably formulated in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form refers to physically discrete units suited as unitary dosages for a mammalian subject; each unit contains a predetermined quantity of active material (*e.g.*, the nucleic acid vaccine) calculated to produce the desired therapeutic effect, in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of, and sensitivity of, ndividual subjects

For lung instillation, aerosolized solutions are used. In a sprayable aerosol preparations, the active protein may be in combination with a solid or liquid inert carrier material. This may also be packaged in a squeeze bottle or in admixture with a pressurized volatile, normally gaseous propellant. The aerosol preparations can contain solvents, buffers, surfactants, and antioxidants in addition to the protein of the invention.

Other pharmaceutically acceptable carriers for the nucleic acid vaccine compositions according to the present invention are liposomes, pharmaceutical compositions in which the active protein is

contained either dispersed or variously present in corpuscles consisting of aqueous concentric layers adherent to lipidic layers. The active protein is preferably present in the aqueous layer and in the lipidic layer, inside or outside, or, in any event, in the non-homogeneous system generally known as a liposomic suspension. The hydrophobic layer, or lipidic layer, generally, but not exclusively, comprises phospholipids such as lecithin and sphingomyelin, steroids such as cholesterol, more or less ionic surface active substances such as dicetylphosphate, stearylamine or phosphatidic acid, and/or other materials of a hydrophobic nature. Those skilled in the art will appreciate other suitable embodiments of the present liposomal formulations.

ANTIGENS ASSOCIATED WITH PATHOGENS

A major use for the present invention is the use of the present nucleic acid compositions in therapeutic vaccine for cancer and for major chronic viral infections that cause morbidity and mortality worldwide. Such vaccines are designed to eliminate infected cells - this requires T cell responses as antibodies are often ineffective. The vaccines of the present invention are designed to meet these needs.

Preferred antigens are epitopes of pathogenic microorganisms against which the host is defended by effector T cells responses, including cytotoxic T lymphocyte (CTL) and delayed type hypersensitivity. These typically include viruses, intracellular parasites such as malaria, and bacteria that grow intracellularly such as Mycobacteria and Listeria species. Thus, the types of antigens included in the vaccine compositions of this invention are any of those associated with such pathogens (in addition, of course, to tumor-specific antigens). It is noteworthy that some viral antigens are also tumor antigens in the case where the virus is a causative factor in cancer.

In fact, the two most common cancers worldwide, hepatoma and cervical cancer, are associated with viral infection. Hepatitis B virus(HBV) (Beasley, R.P. *et al.*, *Lancet* **2**, 1129-1133 (1981) has been implicated as etiologic agent of hepatomas. 80-90% of cervical cancers express the E6 and E7 antigens (exemplified herein) from one of four "high risk" human papillomavirus types: HPV-16, HPV-18, HPV-31 and HPV-45 (Gissmann, L. *et al.*, *Ciba Found Symp.* **120**, 190-207 (1986); Beaudenon, S., *et al. Nature* **321**, 246-249 (1986). The HPV E6 and E7 antigens are the most promising targets for virus associated cancers in immunocompetent individuals because of their ubiquitous expression in cervical cancer. In addition to their importance as targets for therapeutic cancer vaccines, virus associated tumor antigens are also ideal candidates for prophylactic vaccines. Indeed, introduction of prophylactic HBV vaccines in Asia have decreased the incidence of hepatoma (Chang, M.H., *et al. New Engl. J. Med.* **336**, 1855-1859 (1997), representing a great impact on cancer prevention.

Among the most important viruses in chronic human viral infections are HPV, HBV, hepatitis C Virus (HCV), human immunodeficiency virus (HIV-1 and HIV-2), herpesviruses such as Epstein Barr

Virus (EBV),cytomegalovirus (CMV) and HSV-1 and HSV-2 and influenza virus. Useful antigens include HBV surface antigen or HBV core antigen; ppUL83 or pp89 of CMV; antigens of gp120, gp41 or p24 proteins of HIV-1; ICP27, gD2, gB of HSV; or influenze nucleoprotein (Anthony, LS *et al., Vaccine* 1999; 17:373-83). Other antigens associated with pathogens that can be utilized as described herein are antigens of various parasites, includes malaria, preferably malaria peptide (NANP)40.

In addition to its applicability to human cancer and infectious diseases,, the present invention is also intended for use in treating animal diseases in the veterinary medicine context. Thus, the approaches described herein may be readily applied by one skilled in the art to treatment of veterinary herpesvirus infections including equine herpesviruses, bovine viruses such as bovine viral diarrhea virus (for example, the E2 antigen), bovine herpesviruses, Marek's disease virus in chickens and other fowl; animal retroviral and lentiviral diseases (*e.g.*, feline leukemia, feline immunodeficiency, simian immunodeficiency viruses, *etc.*); pseudorabies and rabies; and the like.

As for tumor antigens, any tumor-associated or tumor-specific antigen that can be recognized by T cells, preferably by CTL, can be used. In addition to the HPV-E7 antigen exemplified herein is mutant p53 or HER2/neu or a peptide thereof. Any of a number of melanoma-associated antigens may be used, such as MAGE-1, MAGE-3, MART-1/Melan-A, tyrosinase, gp75, gp100, BAGE, GAGE-1, GAGE-2, GnT-V, and p15 (see, US 6,187,306).

The following references set forth principles and current information in the field of basic, medical and veterinary virology and are incorporated by reference: *Fields Virology*, Fields, BN *et al.*, eds., Lippincott Williams & Wilkins, NY, 1996; *Principles of Virology: Molecular Biology, Pathogenesis, and Control, Flint*, S.J. *et al.*, eds., Amer Society for Microbiology, Washington, 1999; *Principles and Practice of Clinical Virology*, 4th Edition, Zuckerman. A.J. *et al.*, eds, John Wiley & Sons, NY, 1999; *The Hepatitis C Viruses*, by Hagedorn, CH *et al.*, eds., Springer Verlag, 1999; *Hepatitis B Virus: Molecular Mechanisms in Disease and Novel Strategies for Therapy,* Koshy, R. *et al.*, eds,, World Scientific Pub Co, 1998; *Veterinary Virology*, Murphy, F.A. *et al.*, eds., Academic Press, NY, 1999; *Avian Viruses: Function and Control*, Ritchie, B.W., Iowa State University Press, Ames , 2000; *Virus Taxonomy: Classification and Nomenclature of Viruses: Seventh Report of the International Committee on Taxonomy of Viruses,* by M. H. V. Van Regenmortel, MHV *et al.*, eds., Academic Press; NY, 2000.

DELIVERY OF VACCINE NUCLEIC ACID TO CELLS AND ANIMALS

Examples I-III describe certain preferred approaches to delivery of the vaccines of the present invention: naked DNA, self-replicating RNA and virally-based suicide DNA. A broader description of other approaches including viral and nonviral vectors and delivery mechanisms follow.

DNA delivery involves introduction of a "foreign" DNA into a cell *ex vivo* and ultimately, into a live animal or directly into the animal. Several general strategies for gene delivery (= delivery of nucleic acid vectors) for purposes that include "gene therapy" have been studied and reviewed extensively (Yang, N-S., *Crit. Rev. Biotechnol. 12*:335-356 (1992); Anderson, W.F., *Science 256*:808-813 (1992); Miller, A.S., *Nature 357*:455-460 (1992); Crystal, R.G., *Amer. J. Med. 92(suppl 6A)*:44S-52S (1992); Zwiebel, J.A. *et al.*, *Ann. N.Y. Acad. Sci. 618*:394-404 (1991); McLachlin, J.R. *et al.*, *Prog. Nucl. Acid Res. Molec. Biol. 38*:91-135 (1990); Kohn, D.B. *et al.*, *Cancer Invest. 7*:179-192 (1989), which references are herein incorporated by reference in their entirety).

One approach comprises nucleic acid transfer into primary cells in culture followed by autologous transplantation of the *ex vivo* transformed cells into the host, either systemically or into a particular organ or tissue.

The term "systemic administration" refers to administration of a composition or agent such as a molecular vaccine as described herein, in a manner that results in the introduction of the composition into the subject's circulatory system or otherwise permits its spread throughout the body. "Regional" administration refers to administration into a specific, and somewhat more limited, anatomical space, such as intraperitoneal, intrathecal, subdural, or to a specific organ. The term "local administration" refers to administration of a composition or drug into a limited, or circumscribed, anatomic space, such as intratumoral injection into a tumor mass, subcutaneous injections, intramuscular injections. One of skill in the art would understand that local administration or regional administration may also result in entry of a composition into the circulatory system.

For accomplishing the objectives of the present invention, nucleic acid therapy would be accomplished by direct transfer of a the functionally active DNA into mammalian somatic tissue or organ *in vivo*. DNA transfer can be achieved using a number of approaches described below. These systems can be tested for successful expression *in vitro* by use of a selectable marker (*e.g.*, G418 resistance) to select transfected clones expressing the DNA, followed by detection of the presence of the antigen-containing expression product (after treatment with the inducer in the case of an inducible system) using an antibody to the product in an appropriate immunoassay. Efficiency of the procedure, including DNA uptake, plasmid integration and stability of integrated plasmids, can be improved by linearizing the plasmid DNA using known methods, and co-transfection using high molecular weight mammalian DNA as a "carrier".

Examples of successful "gene transfer" reported in the art include: (a) direct injection of plasmid DNA into mouse muscle tissues, which led to expression of marker genes for an indefinite period of time (Wolff, J.A. *et al.*, *Science 247*:1465 (1990); Acsadi, G. *et al.*, *The New Biologist 3*:71 (1991)); (b) retroviral vectors are effective for *in vivo* and *in situ* infection of blood vessel tissues; (c) portal vein

injection and direct injection of retrovirus preparations into liver effected gene transfer and expression *in vivo* (Horzaglou, M. *et al.*, *J. Biol. Chem. 265*:17285 (1990); Koleko, M. *et al.*, *Human Gene Therapy 2*:27 (1991); Ferry, N. *et al.*, *Proc. Natl. Acad. Sci. USA* 88:8387 (1991)); (d) intratracheal infusion of recombinant adenovirus into lung tissues was effective for *in vivo* transfer and prolonged expression of foreign genes in lung respiratory epithelium (Rosenfeld, M.A. *et al.*, *Science 252*:431 (1991); (e) Herpes simplex virus vectors achieved *in vivo* gene transfer into brain tissue (Ahmad, F. *et al.*, eds, *Miami Short Reports - Advances in Gene Technology: The Molecular Biology of Human Genetic Disease*, Vol 1, Boehringer Manneheim1 Biochemicals, USA, 1991).

Retroviral-mediated human therapy utilizes amphotrophic, replication-deficient retrovirus systems (Temin, H.M., *Human Gene Therapy 1*:111 (1990); Temin *et al.*, U.S. Patent 4,980,289; Temin *et al.*, U.S. Patent 4,650,764; Temin *et al.*, U.S. Patent No. 5,124,263; Wills, J.W. U.S. Patent 5,175,099; Miller, A.D., U.S. Patent No. 4,861,719). Such vectors have been used to introduce functional DNA into human cells or tissues, for example, the adenosine deaminase gene into lymphocytes, the NPT-II gene and the gene for tumor necrosis factor into tumor infiltrating lymphocytes. Retrovirus-mediated gene delivery generally requires target cell proliferation for gene transfer (Miller, D.G. *et al.*, *Mol. Cell. Biol. 10*:4239 (1990). This condition is met by certain of the preferred target cells into which the present DNA molecules are to be introduced, *i.e.*, actively growing tumor cells. Gene therapy of cystic fibrosis using transfection by plasmids using any of a number of methods and by retroviral vectors has been described by Collins *et al.*, U.S. Patent 5,240,846.

The DNA molecules encoding the fusion polypeptides of the present invention may be packaged into retrovirus vectors using packaging cell lines that produce replication-defective retroviruses, as is well-known in the art (see, for example, Cone, R.D. *et al.*, *Proc. Natl. Acad. Sci. USA* 81:6349-6353 (1984); Mann, R.F. *et al.*, *Cell* 33:153-159 (1983); Miller, A.D. *et al.*, *Molec. Cell. Biol.* 5:431-437 (1985),; Sorge, J., *et al.*, *Molec. Cell. Biol.* 4:1730-1737 (1984); Hock, R.A. *et al.*, *Nature* 320:257 (1986); Miller, A.D. *et al.*, *Molec. Cell. Biol.* 6:2895-2902 (1986). Newer packaging cell lines which are efficient an safe for gene transfer have also been described (Bank *et al.*, U.S. 5,278,056.

This approach can be utilized in a site specific manner to deliver the retroviral vector to the tissue or organ of choice. Thus, for example, a catheter delivery system can be used (Nabel, EG *et al.*, *Science 244*:1342 (1989)). Such methods, using either a retroviral vector or a liposome vector, are particularly useful to deliver the nucleic acid to be expressed to a blood vessel wall, or into the blood circulation of a tumor.

Other virus vectors may also be used, including recombinant adenoviruses (Horowitz, M.S., In: *Virology*, Fields, BN *et al.*, eds, Raven Press, New York, 1990, p. 1679; Berkner, K.L., *Biotechniques 6*:616 9191988), Strauss, S.E., In: *The Adenoviruses*, Ginsberg, HS, ed., Plenum Press, New York, 1984,

chapter 11), herpes simplex virus (HSV) for neuron-specific delivery and persistence. Advantages of adenovirus vectors for human gene delivery include the fact that recombination is rare, no human malignancies are known to be associated with such viruses, the adenovirus genome is double stranded DNA which can be manipulated to accept foreign genes of up to 7.5 kb in size, and live adenovirus is a safe human vaccine organisms. Adeno-associated virus is also useful for human therapy (Samulski, R.J. *et al., EMBO J. 10*:3941 (1991) according to the present invention.

Another vector which can express the DNA molecule of the present invention, and is useful in the present therapeutic setting, particularly in humans, is vaccinia virus, which can be rendered non-replicating (U.S. Patents 5,225,336; 5,204,243; 5,155,020; 4,769,330; Sutter, G *et al., Proc. Natl. Acad. Sci. USA* (1992) *89*:10847-10851; Fuerst, T.R. *et al., Proc. Natl. Acad. Sci. USA* (1989) *86*:2549-2553; Falkner F.G. *et al.*; *Nucl. Acids Res* (1987) *15*:7192; Chakrabarti, S *et al., Molec. Cell. Biol.* (1985) *5*:3403-3409). Descriptions of recombinant vaccinia viruses and other viruses containing heterologous DNA and their uses in immunization and DNA therapy are reviewed in: Moss, B., *Curr. Opin. Genet. Dev.* (1993) *3*:86-90; Moss, B. *Biotechnology* (1992) *20*:345-362; Moss, B., *Curr Top Microbiol Immunol* (1992) *158*:25-38; Moss, B., *Science* (1991) *252*:1662-1667; Piccini, A *et al., Adv. Virus Res.* (1988) 34:43-64; Moss, B. *et al., Gene Amplif Anal* (1983) 3:201-213.

In addition to naked DNA or RNA, or viral vectors, engineered bacteria may be used as vectors. A number of bacterial strains including *Salmonella*, BCG and *Listeria monocytogenes*(LM) (Hoiseth & Stocker, *Nature* **291**, 238-239 (1981); Poirier, TP *et al. J. Exp. Med.* **168**, 25-32 (1988); (Sadoff, J.C., *et al., Science* **240**, 336-338 (1988); Stover, C.K., *et al., Nature* **351**, 456-460 (1991); Aldovini, A. *et al.,, Nature* **351**, 479-482 (1991); Schafer, R., *et al., J. Immunol.* **149**, 53-59 (1992); Ikonomidis, G. *et al., J. Exp. Med.* **180**, 2209-2218 (1994)). These organisms display two promising characteristics for use as vaccine vectors: (1) enteric routes of infection, providing the possibility of oral vaccine delivery; and (2) infection of monocytes/macrophages thereby targeting antigens to professional APCs.

In addition to virus-mediated gene transfer *in vivo*, physical means well-known in the art can be used for direct transfer of DNA, including administration of plasmid DNA (Wolff *et al.*, 1990, *supra*) and particle-bombardment mediated gene transfer (Yang, N.-S., *et al., Proc. Natl. Acad. Sci. USA 87*:9568 (1990); Williams, R.S. *et al., Proc. Natl. Acad. Sci. USA 88*:2726 (1991); Zelenin, A.V. *et al., FEBS Lett. 280*:94 (1991); Zelenin, A.V. *et al., FEBS Lett. 244*:65 (1989); Johnston, S.A. *et al., In Vitro Cell. Dev. Biol. 27*:11 (1991)). Furthermore, electroporation, a well-known means to transfer genes into cell *in vitro*, can be used to transfer DNA molecules according to the present invention to tissues *in vivo* (Titomirov, A.V. *et al., Biochim. Biophys. Acta 1088*:131 ((1991)).

"Carrier mediated gene transfer" has also been described (Wu, C.H. *et al., J. Biol. Chem. 264*:16985 (1989); Wu, G.Y. *et al., J. Biol. Chem. 263*:14621 (1988); Soriano, P. *et al., Proc. Natl.*

Acad. Sci. USA 80:7128 (1983); Wang, C-Y. *et al.*, *Proc. Natl. Acad. Sci. USA 84*:7851 (1982); Wilson, J.M. *et al.*, *J. Biol. Chem. 267*:963 (1992)). Preferred carriers are targeted liposomes (Nicolau, C. *et al.*, *Proc. Natl. Acad. Sci. USA 80*:1068 (1983); Soriano *et al.*, *supra*) such as immunoliposomes, which can incorporate acylated mAbs into the lipid bilayer (Wang *et al.*, *supra*). Polycations such as asialoglycoprotein/polylysine (Wu *et al.*, 1989, *supra*) may be used, where the conjugate includes a molecule which recognizes the target tissue (e.g., asialoorosomucoid for liver) and a DNA binding compound to bind to the DNA to be transfected. Polylysine is an example of a DNA binding molecule which binds DNA without damaging it. This conjugate is then complexed with plasmid DNA according to the present invention for transfer.

Plasmid DNA used for transfection or microinjection may be prepared using methods well-known in the art, for example using the Quiagen procedure (Quiagen), followed by DNA purification using known methods, such as the methods exemplified herein.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

EXAMPLE I

Enhancement of Sindbis Virus Self-Replicating RNA Vaccine Potency by Linkage of Nucleic Acid encoding *Mycobacterium Tuberculosis* Heat Shock Protein 70 and an Antigen

The present study investigated whether DNA linking full-length E7 to Hsp70 can enhance the potency of self-replicating Sindbis RNA vaccines. We showed that a Sindbis RNA vaccine linking E7 with Hsp70 significantly increased expansion and activation of E7-specific $CD8^+$ T cells and NK cells, bypassing the requirement for CD4+ T cell-mediated help and resulting in potent anti-tumor immunity against E7-expressing tumors. Mechanistic studies confirmed that the Sindbis E7/Hsp70 RNA vaccine induced apoptotic death of host cells and promoted processing of this apoptotic material by dendritic cells (DCs), leading to significantly increased expansion and activation of E7-specific $CD8^+$ T cells. This enhanced CD8 response resulted in a state of potent anti-tumor immunity against an E7-expressing tumor cell line.

MATERIALS AND METHODS

Plasmid DNA Constructs and Preparation

The generation of pcDNA3-Hsp70, pcDNA3-E7, and pcDNA3-E7/Hsp70 has been described previously (Chen *et al.*, *supra*). The Sindbis virus RNA replicon vector, SINrep5 has also been described previously (Bredenbeek, PJ *et al.*, 1993. *J Virol* 67:6439-46). For the generation of SINrep5-

Hsp70, SINrep5-E7, and SINrep5-E7/Hsp70, DNA fragments encoding *Mtb* Hsp70, HPV-16 E7, and chimeric E7/Hsp70 were isolated by cutting pcDNA3-Hsp70, pcDNA3-E7, and pcDNA3-E7/Hsp70 respectively with Xba I and Pme I restriction enzymes, followed by gel recovery from the digested products. These isolated DNA fragments were further cloned into the corresponding Xba I and Pme I sites of the SINrep5 vector to generate SINrep5-Hsp70, SINrep5-E7, and SINrep5-E7/Hsp70 constructs. SINrep5-E7/GFP constructs were generated to evaluate the effect of linkage of E7 to an irrelevant protein. For the generation of SINrep5-E7/GFP, we first constructed pcDNA3-GFP. For the generation of pcDNA3-GFP, a DNA fragment encoding the green fluorescent protein (GFP) was first amplified with PCR using pEGFPN1 DNA (Clontech, Palo Alto, CA) and a set of primers: 5'-atcggatccatggtgagcaagggcgaggag-3' (SEQ ID NO:24) and 5'-gggaagctttacttgtacagctcgtccatg-3' (SEQ ID NO:25). The amplified product was digested with BamHI/ HindIII and further cloned into the BamHI /HindIII cloning sites of pcDNA3 vector. For the generation of pDNA3-E7/GFP, a DNA fragment encoding HPV-16 E7 first amplified with PCR using pcDNA3-E7 as a template and a set of primers: 5'-ggggaattcatgcatggagatacaccta-3' (SEQ ID NO:26) and 5'-ggtggatccttgagaacagatgg-3' (SEQ ID NO:27). The amplified product was then digested with EcoRI/BamHI and further cloned into the EcoRI /BamHI cloning sites of pcDNA3-GFP. E7/GFP was cut with XbaI/PmeI from pcDNA3-E7/GFP and cloned into XbaI/PmeI sites of SIN5rep. The accuracy of these constructs was confirmed by DNA sequencing.

In Vitro RNA Preparation

The generation of RNA transcripts from SINrep5-Hsp70, SINrep5-E7, SINrep5-E7/GFP, SINrep5-E7/Hsp70 and SINrep5 was performed using the protocol described by Mandl *et al.*, (Mandl, CW *et al.*, 1998. *Nat Med* 4:1438-40). SpeI was used to linearize DNA templates for the synthesis of RNA replicons from SINrep5-Hsp70, SINrep5-E7, SINrep5-E7/Hsp70 and SINrep5. RNA vaccines were transcribed *in vitro* and capped using SP6 RNA polymerase and capping analog from the *in vitro* transcription kit (Life Technologies, Rockville, MD) according to vendor's manual. After synthesis, DNA was removed by digestion with DNase I. Synthesized RNA was quantified and analyzed using denaturing formaldehyde agarose gels (Mandl *et al.*, *supra*). The purified RNA was divided into aliquots to be used for vaccination in animals and for transfection of a baby hamster kidney (BHK21) cell line. The protein expression of the transcripts was assessed by transfection of the RNA into BHK21 cells using electroporation.

Cell Lines

BHK21 cells were obtained from the ATCC (Rockville, MD) and grown in Glasgow MEM supplemented with 5% FBS, 10% tryptose phosphate broth, 2 mM glutamine, and antibiotics. Cells

were kept at 37°C in a humidified 5% CO_2 atmosphere and were passaged every 2 days. The production and maintenance of TC-1 cells has been described previously (Lin, KY *et al.,* 1996. *Cancer Research* 56:21-26). On the day of tumor challenge, TC-1 cells were harvested by trypsinization, washed twice with 1X Hanks buffered salt solution (HBSS), and finally resuspended in 1X HBSS to the designated concentration for injection.

ELISA to detect E7 Protein Expression of SINrep5 RNA vaccines

The expression of E7 protein from SINrep5-E7 and SINrep5-E7/Hsp70 RNA was determined by indirect ELISA. The quantity of E7 protein was determined using cell lysates from SIN5rep-E7 or -E7/Hsp70 transfected BHK21 cells. Briefly, 10^7 BHK21 cells were transfected with the 4μg SINrep5, SINrep5-E7, SINrep5-Hsp70, SINrep5-E7/GFP or SINrep5-E7/Hsp70 RNA transcripts respectively via electroporation as described by Liljestrom *et al.,* (Liljestrom, PS *et al.,* 1991. *J Virol* 65:4107-13). We used SINrep5-vector containing the β-gal gene and determined the transfection efficiency. The transfected cells were fixed and stained for lacZ expression using X-Gal (Sanes, JR *et al.,* 1986. *Embo J* 5:3133-42). In general, the transfection efficiency in our electroporation was consistent and measured around 30%. The transfected BHK21 cells were collected 16-20 hrs after electroporation. A 96-microwell plate was coated BHK 21 cell lysates that were transfected with various SINrep5 RNA constructs in a final volume of 100 μl, and were incubated at 4°C overnight. The bacteria-derived HPV-16 E7 proteins were used as a positive control. The wells were then blocked with PBS containing 20% fetal bovine serum. Diluted anti-E7 Ab (Zymed, San Francisco, CA) were added to the ELISA wells, and incubated on 37°C for 2 hr. After washing with PBS containing 0.05% Tween-20, the plate was incubated with 1/2000 dilution of a peroxidase-conjugated rabbit anti-mouse IgG antibody (Zymed, San Francisco, CA) at room temperature for one hour. The plate was washed, developed with 1-Step™ Turbo TMB-ELISA (Pierce, Rockford, IL), and stopped with 1M H_2SO_4. The ELISA plate was read with a standard ELISA reader at 450 nm. The quantity of E7 protein of the cell lysates was then calculated and determined by comparing with the standardized E7 protein.

Mice

6 to 8-week-old female C57BL/6 mice from the National Cancer Institute (Frederick, MD) were purchased and kept in the oncology animal facility of the Johns Hopkins Hospital (Baltimore, MD). All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

RNA Vaccination

All SINrep5 RNA vaccines were generated using *in vitro* transcription as described earlier. RNA concentration was determined by optical density measured at 260 nm. The integrity and quantity of RNA transcripts were further checked using denaturing gel electrophoresis. Mice were vaccinated intramuscularly with 10 μg of SINrep5-Hsp70, SINrep5-E7, SINrep5-E7 mixed with SINrep5-Hsp70, SINrep5-E7/GFP or SINrep5 RNA vaccines in the right hind leg while SINrep5-E7/Hsp70 was administered in 0.1, 1, and 10 μg quantities.

ELISA to Measure Anti-E7 Antibodies

Anti-HPV 16 E7 antibodies in the sera were determined by direct ELISA as described previously (Wu, TC 1995. *Proc Natl Acad Sci USA* 92:11671-5). A 96-microwell plate was coated with 100 μl bacteria-derived HPV-16 E7 proteins (5 μg/ml) and incubated at 4°C overnight. The wells were then blocked with PBS containing 20% fetal bovine serum. Sera obtained from mice on day 14 post-immunization were serially diluted in PBS, added to the ELISA wells, and incubated at 37°C for 2 hr. After washing with PBS containing 0.05% Tween-20, the plate was incubated with 1/2000 dilution of a peroxidase-conjugated rabbit anti-mouse IgG antibody (Zymed, San Francisco, CA) at room temperature for one hour. The plate was washed, developed with 1-Step™ Turbo TMB-ELISA (Pierce, Rockford, IL), and stopped with 1M H_2SO_4. The ELISA plate was read with a standard ELISA reader at 450 nm.

ELISA to Measure IFNγ

Splenocytes were harvested 2 weeks after vaccination and cultured with the E7 peptide (aa 49-57) containing the MHC class I epitope (RAHYNIVTF, SEQ ID NO:22) (Feltkamp, MC *et al.*, 1993. *Eur J Immunol* 23:2242-9) or the E7 peptide (aa 30-67) containing the class II epitope (DSSEEEDEIDGPAGQAEPDRAHYNIVTFCCKCDSTLRL; SEQ ID NO:23) (Tindle, RW 1991. *Proc Natl Acad Sci USA* 88:5887-91) in a total volume of 2 ml of RPMI 1640, supplemented with 10% (vol/vol) fetal bovine serum, 50 units/ml penicillin and streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mM nonessential amino acids in a 24-well tissue culture plate for 6 days. The supernatants were harvested and assayed for the presence of IFNγ using a commercial ELISA kit (Endogen, Woburn, MA) according to the manufacturer's protocol.

Cytotoxic T Lymphocyte (CTL) Assays

Cytolysis was determined by quantitative measurements of lactate dehydrogenase (LDH) released from cells using CytoTox96®, a non-radioactive cytotoxicity assay kit (Promega, Madison, WI) according to the manufacturer's protocol. Briefly, splenocytes were harvested and pooled 2 weeks after RNA vaccination. Five mice were used for each vaccinated group. Splenocytes were cultured with the E7 peptide (aa 49-57) in a total volume of 2 ml of RPMI 1640, supplemented with 10% (vol/vol) fetal

bovine serum, 50 units/ml penicillin/ streptomycin, 2mM L-glutamine, 1mM sodium pyruvate, 2mM nonessential amino acids in a 24-well tissue culture plate for 6 days as effector cells. TC-1 tumor cells were used as target cells. The TC-1 cells mixed with splenocytes at various effector/target (E/T) ratios. After 5 hr incubation at 37°C, 50µl of the culture medium was collected to assess the amount of LDH present. The percentage of lysis was calculated from the formula: 100 x {(A-B)/(C-D)}, where A is the reading of experimental-effector signal value, B is the effector spontaneous background signal value, C is maximum signal value from target cells, D is the target spontaneous background signal value.

In Vivo Tumor Protection Experiments

For the tumor protection study, mice (5 per group) were immunized i.m. with 10µg/mouse of SINrep5-Hsp70, SINrep5-E7, SINrep5-E7 mixed with SINrep5-Hsp70, SINrep5-E7/GFP or SINrep5 RNA, or 0.1µg/mouse, 1 µg/mouse, or 10µg/mouse of SINrep5-E7/Hsp70 RNA. 14 days after immunization, mice were injected intravenously with 1x 10^4 cells/mouse TC-1 tumor cells in the tail vein. Three weeks after tumor challenge, mice were euthanized. The number of tumor nodules on the lung surface in each mouse was evaluated and counted by experimenters in a blinded fashion.

In Vivo Depletion of Cells using Monclonal Antibodies

The procedure was described previously (Lin, KY *et al.,* 1996. *Cancer Research 56:21-26;* Wu, TC *et al.,* 1995. *J. Exp. Med 182:1415-1421*). In brief, each mouse was vaccinated with 1 µg self-replicating SINrep5-E7/Hsp70 RNA i.m. and challenged with 10^4 TC-1 tumor cells i.v. (via tail vein). Depletions were initiated one week prior to tumor challenge. IgG_{2a} antibody (PharMingen, San Diego, CA) was used as a non-specific isotype control. MAb GK1.5 (Dialynas, DP 1983. *J. Immunol. 131:2445*) was used for depletion of CD4+ cells; mAb 2.43 (Sarmiento, MA *et al.,* 1980. *J. Immunol. 125:2665*) was used for depletion of CD8+ cells; and mAb PK136 (Koo, GC *et al., J Immunol. 137:3742*) was used for depletion of NK1.1+ cells. Flow cytometry analysis revealed that >95% of the cells of the appropriate lymphocyte subset were depleted while numbers of cells of other subsets were unchanged. Depletion treatment was discontinued on day 21 after tumor challenge.

Cell Surface Marker Staining and Flow Cytometric Analysis

Splenocytes from naïve or vaccinated mice were immediately stained for cell surface markers according to Ji, H *et al.,* 1999, *Human Gene Therapy* 10:2727-2740. Cells were then washed once in FACScan® buffer and stained with PE-conjugated monoclonal rat anti-mouse NK1.1 antibody or FITC-conjugated monoclonal rat anti-mouse CD3 antibody (PharMingen, San Diego, CA). NK cells are NK1.1+ and CD3-negative. Flow cytometry was used to determine the percent of splenocytes that were NK cells.

In Vitro Analysis of Cell Death

10^7 BHK21 cells were transfected with 4 μg SINrep5, SINrep5-E7, SINrep5-Hsp70 or SINrep5-E7/Hsp70 RNA transcripts as mentioned above. The transfection efficiency was around 20-30%. Native BHK21 cells or BHK21 cells that were electroporated without SINrep5 RNA served as controls. BHK21 cells were collected and assessed every 24 hr, until hour 72. The percentages of apoptotic and necrotic BHK21 cells were determined using annexin V apoptosis detection kits (PharMingen, San Diego, CA) according to the manufacturer's protocol, followed by flow cytometry.

Generation and Culture of Dendritic Cells (DCs) from Bone Marrow

DCs were generated by culturing bone marrow cells in the presence of GM-CSF as described previously (Lu, Z *et al.*, 2000. *J Exp Med* 191:541-550). Briefly, bone marrow was collected from the tibias of mice. Erythrocytes were lysed, and the remaining cells were passed through a nylon mesh to remove small pieces of bone and debris. The cells were collected and 10^6 cells/ml were placed in 24-well plates in RPMI 1640, supplemented with 5% FCS, 2mM β-mercaptoethanol, 1% nonessential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Rockville, MD), and 100 U/ml GM-CSF (PharMingen, San Diego, CA). Two-thirds of the medium was replaced every 2 days, and nonadherent cells were harvested on day 7. The collected cells were characterized by flow cytometry for DC markers as previously described (Wang, TL *et al.*, 2000. *J Exp Med* 191:541-550).

CTL Assay Using DCs Pulsed with BHK21 Cells that had been Transfected with Various RNA Transcripts as Target Cells

CTL assays using DCs pulsed with BHK21 cells that had been transfected with various RNA transcripts as target cells were performed using a protocol similar to that described by Albert, ML *et al.*, 1998. *Nature 392:86-9* and Albert ML, *et al.*, 1998. *J Exp Med 188:1359-68*). Briefly, 10^7 BHK21 cells were transfected with 4μg of various self-replicating SINrep5 RNA constructs via electroporation. The cells were collected 16-20 hr later. The levels of E7 protein expression in BHK21 cells transfected with SINrep5-E7, or SINrep5-E7/Hsp70 RNA transcripts were similar, as determined by ELISA. 3 x 10^5 transfected BHK21 cells were co-incubated with 10^5 of bone marrow-derived DCs at 37°C for 48 hr. These "prepared" DCs were then used as target cells (T) and the H-2D^b-restricted E7-specific CD8+ T cells were used as effector cells (E) (Wang, *et al., supra*). CTL assays were performed with 10^4 target cells and effector cells numbers yielding E/T ratios of 1, 3 and 9, incubated in a final volume of 200 μl. After 5 hrs at 37°C, 50 μl of culture supernatant were collected to assess the amount of LDH as described above. Negative controls included DCs co-incubated with untransfected BHK21 cells, transfected BHK21 cells incubated alone, untreated DCs incubated alone, and cells of the CD8+ T cell line incubated alone.

RESULTS

Construction and Characterization of Self-replicating RNA Constructs

Generation of plasmid DNA constructs and subsequent preparation of self-replicating SINrep5 RNA constructs was performed as described above. The SINrep5 vector includes DNA encoding Sindbis virus RNA replicase and the SP6 promoter ((Bredenbeek, PJ *et al.,* 1993. *J Virol* 67:6439-46)). A schematic diagram of SINrep5, SINrep5-Hsp70, SINrep5-E7, SINrep5-E7/GFP and SINrep5-E7/Hsp70 RNA transcripts using SP6 RNA polymerase is shown in Figure 1. An ELISA to test expression of E7 protein by BHK21 cells transfected with the various self-replicating RNA constructs showed that SINrep5-E7 and SINrep5-E7/Hsp70 expressed similar amounts of E7 protein.

Vaccination with Self-replicating SINrep5-E7/Hsp70 RNA Enhances an E7-Specific Cytotoxic T cell Response

$CD8^+$ T lymphocytes are important effectors of anti-tumor immunity. Generation of E7-specific $CD8^+$ CTLs following vaccination was assessed. Figure 2 shows results of a study wherein splenocytes from the various self-replicating SINrep5 RNA vaccines were cultured with the E7 peptide (aa 49-57) for 6 days and were examined as effector cells against TC-1 tumor cell targets. The self-replicating SINrep5-E7/Hsp70 generated a significantly greater lytic activity in the lymphocyte population compared to cells from mice vaccinated with the other SINrep5 RNA vaccines ($p<0.001$, one-way ANOVA). The capacity of SINrep5-E7/Hsp70 RNA to generate lytic activity was approximately 7 times that induced by self-replicating SINrep5-E7 RNA (57.2±6.8 % versus 8.0±1.3 %, E/T ratio 45, $p<0.001$).

The concentration of IFN-γ in the supernatant of stimulated splenocytes was assessed by ELISA. Splenocytes from mice given the various self-replicating RNA vaccines were cultured *in vitro* with E7 peptide (aa 49-57) (MHC class I epitope (Feltkamp *et al., supra*)) for 6 days. As a negative control, the stimulatory peptide was omitted. Culture supernatants were collected for measurement of IFN-γ concentration. As shown in Figure 3, peptide-stimulated splenocytes from mice vaccinated with self-replicating E7/Hsp70 RNA secreted the highest concentration of IFN- γ compared to cells from mice given the other RNA vaccines ($p<0.001$, one-way ANOVA). Fusion of Hsp70 to E7 significantly enhanced IFN-γ-secreting E7-specific $CD8^+$ T cell activity.

Vaccination with Self-replicating SINrep5-E7/Hsp70 RNA Did Not Enhance IFN-γ-secreting E7-Specific $CD4^+$ T cells or Anti-E7 Antibodies

ELISA was used to assess the E7-specific $CD4^+$ T cell responses generated by the vaccines by measuring concentration of IFN-γ in the supernatant of cultured splenocytes. Splenocytes were cultured in the presence of with E7 peptide (aa 30-67) (that includes an MHC class II epitope (Tindle *et al.*, *supra*) for 6 days. The peptide was omitted in the negative control. As shown in Figure 4, there was no

significant increase in the concentration of IFN-γ from splenocytes obtained from mice vaccinated with self-replicating E7/Hsp70 RNA compared to the other RNA vaccines. Therefore, a vaccine in which Hsp70 is fused to E7 does appear to enhance IFN-γ-secreting E7-specific $CD4^+$ T cell activity.

The quantity of anti- E7 antibodies in the sera of the vaccinated mice was determined y direct ELISA two weeks after vaccination. Sera of mice vaccinated with SINrep5-E7/Hsp70 did have higher titers of E7-specific antibodies compared to mice vaccinated with other RNA vaccine constructs.

Vaccination with Self-Replicating SINrep5-E7/Hsp70 RNA Protects Mice Better Against the Growth of E7-Expressing TC-1 Tumors

An *in vivo* tumor protection experiment was performed using different doses of SINrep5-E7/Hsp70 RNA administered intramuscularly in the right hind leg. Each mouse was vaccinated with 10 μg of one of the following constructs: self-replicating SINrep5, SINrep5-E7, SINrep5-Hsp70, SINrep5-E7 mixed with SINrep5-Hsp70, SINrep5-E7/GFP or SINrep5-E7/Hsp70 RNA. Self-replicating E7/Hsp70 RNA was also tested at doses of 0.1 and 1 μg/mouse. E7-expressing TC-1 cells were injected i.v. 14 days later. Such tumor challenge simulates hematogenous spread of the tumor cells, allowing evaluation of vaccine effects on metastasis to the lungs via he bloodstream. Pulmonary nodules were assessed 21 days after tumor challenge. Figure 5 shows a lower mean number of pulmonary nodules in mice vaccinated with the self-replicating E7/Hsp70 RNA vaccines (0.1 μg, 1μg, and 10 μg) compared to mice given the other RNA vaccines ($p<0.001$, one-way ANOVA). Representative photographs of the lung tumors (unmagnified) are shown in Figure 6. The results demonstrated that self-replicating RNA SINrep5-E7/Hsp70 vaccines protected mice from i.v. tumor challenge even at the lower dose of 0.1 μg whereas vaccination with 10 μg of SINrep5 without insert, SINrep5-E7, SINrep5-Hsp70, SINrep5-E7 mixed with SINrep5-Hsp70, or SINrep5-E7/GFP RNA showed no or little protection, developing numerous tumor nodules. These results also showed that linkage of RNA encoding E7 to RNA encoding an irrelevant protein such as GFP did not result in an antitumor effect, but rather that and that antitumor protection offered by Hsp70 required physical linkage of E7 to Hsp70 at the nucleic acid level.

$CD8^+$ T Cells and NK cells Are Important for the Anti-tumor Effect

To determine the types of lymphocytes required for protection against E7-expressing tumors, *in vivo* antibody depletion experiments were done in which depletion was initiated one week before tumor challenge and terminated on day 21 after tumor challenge. As shown in Figure 7, the mean number of pulmonary nodules from mice depleted of $CD8^+$ T cells or of NK1.1+ cells was significantly higher than that observed in mice treated with control IgG2a isotype antibody (which was similar to no antibody depletion). Furthermore, depletion of NK1.1+ cells resulted in a higher number of tumor lung nodules than depletion of $CD8^+$ cells. In contrast, the mean number of pulmonary nodules from mice depleted of

$CD4^+$ T cells resembled the isotype controls, indicating that $CD4^+$ T cells were not critical for this effect. Therefore, it was concluded that (Hariharan *et al., supra*). $CD8^+$ T cells are essential for generation of antigen-specific anti-tumor immunity by SINrep5-E7/Hsp70 RNA vaccine and (Berglund, PM *et al.*, 1997. *AIDS Res Hum Retroviruses* 13:1487-95) NK cells play an important role as well.

To investigate whether NK cells were significantly expanded in mice vaccinated with various RNA constructs, flow cytometry analysis was performed, evaluating CD3(-), NK1.1+ cells. The proportion of NK cells was markedly increased in mice vaccinated with each of the all constructs (E7/Hsp70, E7, Hsp70, and control plasmid) relative to naïve mice, indicating that the expansion of NK cells is not a response limited to the E7/Hsp70 vaccine (Figure 8).

Self-Replicating RNA Vaccines Induce Apoptosis

Self-replicating RNA vaccines have been shown to induce apoptotic changes following uptake by cells (Ying *et al., supra*). We evaluated apoptosis in BHK21 cells transfected with various RNA vaccines. Percentages of apoptotic BHK21 cells were normalized for transfection efficiency. As shown in Figure 9, apoptosis was induced in all groups of BHK21 cells transfected with various of the RNA vaccines compared to two negative control groups (untransfected or electroporated without RNA). There were no significant difference between the different RNA vaccines. there A steady decline in apoptosis occurred from 24 hr to 72 hr after transfection (with SIN5-E7/Hsp70: 70.3±3.6% at 24 hr, 49.3±4.2% at 48 hr, 18.0±3.1% at 72 hr, $p<0.001$, one-way ANOVA). These results confirm that cells transfected with each of these self-replicating RNA vaccines undergo apoptotic changes.

Enhanced Presentation of E7 through the MHC Class I Pathway in Dendritic Cells Pulsed With Cells Transfected with SINrep5-E7/Hsp70 RNA

Enhanced E7-specific $CD8^+$ T cell responses *in vivo* may occur as a result of presentation of E7 via the MHC class I pathway resulting from uptake of apoptotic cellular material expressing various E7 constructs by host APCs. An experiment was performed to characterize the MHC class I presentation of E7 in DCs "pulsed" with BHK21 cells that had been transfected with various self-replicating RNA constructs. As noted above, the transfection efficiency and E7 expression is similar in BHK21 cells transfected with the various E7-containing self-replicating RNAs. Here, transfected BHK21 cells were incubated with bone marrow-derived DCs to allow antigen uptake and processing by the DCs. These DCs were then used as target cells for killing by E7-specific $CD8^+$ CTL. As shown in Figure 10, DCs incubated with BHK21 cells that had been transfected with SINrep5-E7/Hsp70 RNA were lysed to a higher degree than DCs incubated with BHK21cells transfected with SINrep5-E7 RNA ($p<0.001$). These results suggested that the presence of Hsp70 in a fusion protein with E7 (that was "fed" to DCs in

the form of RNA-transfected BHK21 cells) resulted in more effective presentation of E7 by DCs (via the MHC class I pathway) to CD8+ T cells as compared to E7 protein alone.

DISCUSSION

A vaccine designated SINrep5-E7/Hsp70, administered *in vivo* significantly enhanced E7-specific $CD8^+$ T cell responses compared to the SINrep5-E7 RNA vaccine lacking Hsp70. It is unlikely that this effect results from occurs improved direct MHC class I presentation of E7 to CTLs by the cells that actually express E7/Hsp70 -- a process known as "direct priming". Intramuscular delivery of RNA replicons is believed to deliver RNA into muscle cells, which are not "professional" APCs because they do not express co-stimulatory molecules that are important for efficient activation of T cells. Even if the various SINrep5 constructs are delivered to other cell types after i.m. administration, the self-replicating RNA eventually causes apoptosis of the cells it transfects (Frolov *et al.*, *supra*). The cell initially transfected are therefore unlikely to be efficient direct presenters of antigen.

Rather, the enhanced $CD8^+$ T cell responses are likely a result of a process whereby apoptotic cells (and subcellular material) are endocytosed and processed by professional APCs via MHC class I pathways for more effective presentation to $CD8^+$ T cells (Albert *et al.*, *supra*) Alternatively, apoptotic cells may release the chimeric E7/Hsp70 protein which is then taken up and processed by APCs via the MHC class I-restricted pathway (Srivastava, PK *et al., Immunogenetics 39:93-8*; Arnold, D *et al.,* 1995. *J Exp Med 182:885-9*; Suto, R *et al.,* 1995. *Science 269:1585-8*). Hsp70 complexes are known to enter professional APCs by binding specifically to the cell surface followed by receptor-mediated endocytosis (Arnold-Schild, D *et al.,* 1999. *J Immunol* 162:3757-60). In recent investigations of receptors for heat shock proteins, CD91 was identified as the receptor for gp96, one member of the HSP family on APCs (Binder, RJ *et al.,* 2000 *Nat. Immuno.l* 2:151-155).

Another important factor for enhanced activation of antigen specific $CD8^+$ T cells by chimeric Hsp70/E7 may be the biology of professional APCs, primarily DCs. Cho *et al.,* recently reported that a mycobacterial HSP fused to an antigen stimulates DCs to upregulate expression of MHC class I, class II and co-stimulatory molecules (Cho, BK 2000. *Immunity* 12:263-272). Thus, induction of DC "maturation" by Hsp70 linked to antigen may augment T cell activity, explaining the results described herein with the chimeric E7/Hsp70 RNA vaccine.

The present study demonstrated that depletion of NK cells reduced the antitumor effect induced by the E7/Hsp70 RNA replicon-based vaccine (Figure 7), indicating that these cells are a necessary component. However, NK cell activity alone cannot account for the observed antitumor effect because other of the RNA replicon-based compositions produced a similar change in NK cell number (Figure 8). The *in vivo* antibody depletion study suggested that $CD8^+$ CTLs were important for this antitumor effect

(Figure 7). Thus, it was concluded that both NK cells and $CD8^+$ T cells are important contributors to the antitumor effect of the E7/Hsp70 RNA vaccine. Interactions among these two cell populations might also be of interest in understanding the outcome of such vaccination.

A comparison of the study described above with previous studies of the present inventors and their colleagues reveals that different forms of nucleic acid vaccines may activate different subsets of effector cells in the vaccinated host and act via different mechanisms.

Even though NK cells appeared to play a role in the anti-tumor effects induced by E7/Hsp70 RNA replicons, NKs were not essential when the vaccine was a naked E7/Hsp70 DNA vaccine. Thus,, depletion of $NK1.1^+$ cells in mice vaccinated with naked E7/Hsp70 DNA did not decrease the anti-tumor immunity (Chen *et al.*, *supra*).

In contrast, $CD8^+$ T cells were important for antitumor effects induced by both E7/Hsp70 DNA and E7/Hsp70 RNA replicon-based vaccines.

The apoptotic changes promoted by the self-replicating RNA vaccine raise potential safety concerns. With RNA replicon-based vaccines, increased apoptotic changes and inflammatory responses are localized to the injection sites. However, microscopic examination of the vital organs of E7/Hsp70-vaccinated mice did not show any significant histopathological changes. Potential risks attend the presence of HPV-16 E7 protein in host cells as E7 happens to be a viral oncoprotein that disrupts cell cycle regulation by binding to tumor suppressor pRB protein in nuclei (Lukas, JH *et al.*, 1994. *J Cell Biol* 125:625-38), leading to potential incidence and accumulation of genetic aberrations and eventual malignant transformation. Use of a self -replicating RNA vector eases the concern about oncogenicity of E7 protein since the transfected cells eventually undergo apoptosis.

In summary, the results reveal that fusion of DNA encoding *Mtb* Hsp70 to DNA encoding HPV-16 E7 in an RNA replicon results in a vaccine composition that induces a marked antigen (E7)-specific $CD8^+$ T cell-mediated immune response that produces a state of anti-tumor immunity against tumors expressing the antigen. Fusion of Hsp70 DNA to DNA encoding an antigen further enhances the potency of the RNA replicon-based vaccine. These findings are applicable to other tumors and types of cancer where tumor-specific antigens can be identified. Further, these findings are directly applicable to vaccines against organisms responsible for infectious diseases such as viruses, protozoa, fungi and bacteria.

EXAMPLE II

Enhancement of Suicidal DNA Vaccine Potency by Linking *Mycobacterium Tuberculosis* Heat Shock Protein 70 to an Antigen

Recently, RNA replicon vaccines have emerged as an important strategy to alleviate the concerns for potential chromosomal integration and cell transformation noted above (Ying *et al., supra*). These vaccines are self-replicating, self-limiting and may be administered either as RNA or as DNA which is then transcribed into RNA replicons in transfected cells *in vitro* or *in vivo*. DNA-based self-replicating RNA replicons, also known as "suicidal DNA," eventually cause lysis of transfected cells(Berglund *et al., supra*; Leitner *et al., supra*).

The present vaccine was developed using the Semliki Forest virus suicidal DNA vector, pSCA1 (DiCiommo, DP *et al., J Biol Chem* 1998; 273:18060-6). Such vectors alleviate some concern about naked DNA because they eventually cause apoptosis of transfected cells. This feature is particularly desirable for vaccines that encode potentially oncogenic proteins, such as the HPV E7 protein (Wu, TC. *Curr Opin Iminunol* 1994; 6:746-754). Because suicidal DNA vectors eventually kill transfected cells, any expression of DNA from these vectors is necessarily transient, conceivably compromising their potency. Therefore, the present inventors have conceived of strategies to enhance the potency of suicidal DNA vaccines.

Disclosed herein are the findings of an investigation of the impact of linking full-length Hsp70 to E7 on the potency of suicidal DNA vaccines. The suicidal DNA vaccine, pSCA1-E7/Hsp70, significantly increased expansion and activation of E7-specific $CD8^+$ T cells compared to a vaccine comprising only pSCA1-E7. This enhanced response resulted in potent anti-tumor immunity against E7-expressing tumor cells.

Materials and Methods

Plasmid DNA Constructs and Preparation

pSCA1 vector (DiCiommo *et al., supra*) was a gift from Dr. Bremner at the University of Toronto. This vector contains the human CMV immediate early gene (HCMV IE) promoter upstream of the Semliki Forrest virus (SFV) replicon. The subgenomic promoter is located after the SFV replicon, upstream of a multiple cloning sites for insertion of DNA of interest. For the generation of pSCA1-E7, E7 was cut from pcDNA3-E7 by BamHI/PmeI (Chen *et al., supra*) and cloned into BamHI/SmaI sites of pSCA1. To construct pSCA1-Hsp70, Hsp70 was cut from pcDNA3-Hsp70 [8]by BamHI/PmeI and cloned into BamHI/SmaI sites of pSCA1. For the generation of pSCA1-E7/Hsp70, E7/Hsp70 DNA was cut from pcDNA3-E7/Hsp70 (Chen *et al., supra*) by BamHI/PmeI and cloned into BamHI/SmaI sites of

pSCA1. The accuracy of these constructs was confirmed by DNA sequencing. Plasmid DNA vectors encoding pSCA1-E7, pSCA1-Hsp70, pSCA1-E7/Hsp70 or pSCA1 with no insert were transfected into subcloning efficient DH5™ cells (Life Technologies, USA). The DNA was then amplified and purified (Chen *et al.*, *supra*). The integrity of plasmid DNA and the absence of *Escherichia coli* DNA or RNA were checked in each preparation using 1% agarose gel electrophoresis. DNA concentration was determined by optical density measured at 260 nm. The presence of the inserted E7 fragment was confirmed by restriction enzyme digestion and gel electrophoresis.

Cell Lines

The production and maintenance of TC-1 cells has been described previously (Lin *et al.*, *supra*). In brief, HPV-16 E6, E7 and *ras* oncogene DNA were used to transform primary lung epithelial cells from C57BL/6 mice. The cells were grown in RPMI 1640, supplemented with 10% (vol/vol) fetal bovine serum, antibiotics, L-glutamine, sodium pyruvate, nonessential amino acids at 37°C with 5% CO_2. On the day of tumor challenge, TC-1 cells were harvested by trypsinization, washed twice with 1X Hanks buffered salt solution (HBSS) and finally resuspended in 1X HBSS to the designated concentration for injection.

Mice

6- to 8-week old female C57BL/6 mice from the National Cancer Institute (Frederick, MD) were purchased and kept in the oncology animal facility of the Johns Hopkins Hospital (Baltimore, MD). All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

DNA Vaccination

Gene gun particle-mediated DNA vaccination was performed using a helium-driven gene gun (Bio-rad, Hercules, CA) according to the protocol provided by the manufacturer. Briefly, DNA coated gold particles were prepared by combining 25 mg of 1.6 μm gold microcarriers (Bio-rad, Hercules, CA) and 100 μl of 0.05 M spermidine (Sigma, St, Louis, MO). Plasmid DNA (50 μg) and 1.0 M $CaCl_2$ (100 μl) were added sequentially to the microcarriers while mixing by vortex. This mixture was allowed to precipitate at room temperature for 10 minutes. The microcarrier/DNA suspension was then centrifuged (10,000 rpm. for 5 sec) and washed 3 times in fresh absolute ethanol before resuspending in 3 ml of polyvinylpyrrolidone (0.1 mg/ml) (Bio-rad, Hercules, CA) in absolute ethanol. The solution was then loaded into tubing and allowed to settle for 4 min. The ethanol was gently removed and the microcarrier/DNA suspension was evenly attached to the inside surface of the tubing by rotating the tube. The tube was then dried using 0.4 liters per minute of flowing nitrogen gas. The dried tubing coated with microcarrier/DNA was then cut to 0.5-inch cartridges and stored in a capped dry bottle at

4°C. As a result, each cartridge contained 1 μg of plasmid DNA and 0.5 mg of gold. The DNA coated gold particles (1 μg DNA/bullet) were delivered to the shaved abdominal region of the mice using a helium-driven gene gun (Bio-rad, Hercules, CA) with a discharge pressure of 400 p.s.i.

Intracytoplasmic Cytokine Staining and Flow Cytometry Analysis

Splenocytes from groups naïve or vaccinated mice (5 mice per group) were collected and pooled one week after the last vaccination and incubated either with the E7 peptide (aa 49-57, RAHYNIVTF; SEQ ID NO:22) containing MHC class I epitope or the E7 peptide (aa 30-67) containing MHC class II peptide. The E7 peptide was added at a concentration of 2 μg/ml for 20 hours. To detect E7-specific $CD8^+$ T cell precursors and E7-specific $CD4^+$ T helper cell responses, $CD8^+$ CTL epitopes aa 49-57 or aa 30-67 of E7 were used, respectively. Golgistop (Pharmingen, San Diego, CA) was added 6 hr before harvesting the cells from the culture. Cells were then washed once in FACScan buffer and stained with phycoerythrin (PE)-conjugated monoclonal rat anti-mouse CD8 or CD4 antibody (Pharmingen, San Diego, CA). Cells were subjected to intracellular cytokine staining using the Cytofix/Cytoperm kit according to the manufacturer's instructions (Pharmingen). FITC-conjugated anti-IFN-γ or anti-IL-4 antibodies and the immunoglobulin isotype control antibody (rat IgG1) were all purchased from Pharmingen. Analysis was done on a Becton-Dickinson FACScan flow cytometer with CELLQuest software (Becton Dickinson Immunocytometry System, Mountain View, CA).

ELISA for anti-E7 antibody (see EXAMPLE I)

In vivo Tumor Protection

These studies were generally as Example I, except that different DNA preparations and deliver was used: gene gun with 2 μg of pSCA1-E7, pSCA1-Hsp70, pSCA1-E7/Hsp70 DNA, or pSCA1 without insert. One week later, mice were boosted with the same regimen and, on day 14, were challenged subcutaneously with 10^4 TC-1 tumor cells in the right leg.

Tumor Therapy

The tumor cells and DNA vaccines were prepared as above. Each mouse (5 per group) was challenged i.v. with 10^4 TC-1 tumor cells on day 0. Three days later, mice were given 2 μg of a vaccine preparation (pSCA1-E7, pSCA1-Hsp70, pSCA1-E7/Hsp70 DNA, or pSCA1 without insert) via gene gun. One week later, animals were boosted using the same regimen and were sacrificed on day 21. The number of tumor nodules on the surface of the lung of each mouse were evaluated and counted by experimenters blinded to sample identity. Statistical significance was tested using one-way ANOVA.

In vivo Antibody Depletion Experiments

The procedure was done as in Example I. Here, vaccination was with 2 μg DNA via gene gun, boosted one week later, and challenged with 5 x 10^4 TC-1 tumor cells. Depletion treatment was terminated 40 days after tumor challenge.

RESULTS

Construction and Characterization of the pSCA1-E7/Hsp70 Suicidal DNA Vaccine

The generation of plasmid DNA constructs and subsequent preparation of DNA-based self-replicating pSCA1 vaccines was performed as described above. The pSCA1 vector includes the HCMV IE promoter and a replicon from the SFV (DiCiommo *et al, supra*). A schematic diagram depicting DNA-based self-replicating pSCA1-E7, pSCA1-Hsp70, and pSCA1-E7/Hsp70 constructs is shown in **Figure 11.**

An ELISA to test expression of E7 protein by BHK21 cells transfected with the various DNA-based self-replicating E7-containing pSCA1 DNA constructs showed that similar amounts of E7 protein were expressed by each of these constructs.

Vaccination with the pSCA1-E7/Hsp70 Suicidal DNA Vaccine Enhances E7-Specific $CD8^+$ T Cell-mediated Immune Responses

$CD8^+$ T lymphocytes are important effectors of anti-tumor immunity. As a measure of the E7-specific $CD8^+$ T cell response generated by the DNA-based self-replicating pSCA1-E7/Hsp70 vaccine, intracellular cytokine staining was evaluated in splenocytes from mice vaccinated intradermally via gene gun. As shown in **Figure 12A**, vaccination of mice with pSCA1-E7/Hsp70 suicidal DNA vaccine generated the highest number of E7-specific IFN-γ^+ $CD8^+$ T cell precursors (40 per $3x10^5$ splenocytes) compared to vaccination with pSCA1-E7 DNA (12 per $3x10^5$ splenocytes) ($p<0.01$). pSCA1-E7/Hsp70 DNA immunization led to a nearly 4-fold increase in the number of E7-specific $CD8^+$ T cell precursors. The mean number of IFN-γ-producing E7-specific $CD8^+$ T cells was determined in the presence (solid columns) and absence (open columns) of E7 peptide aa 49-57(SEQ ID NO:22) and shown in **Figure 12B**. These results indicated that linkage of Hsp70 to E7 significantly enhanced the frequency of E7-specific $CD8^+$ T cell precursors in vaccinated mice.

Vaccination with pSCA1-E7/Hsp70 Suicidal DNA Vaccine Did not Induce Significant E7-Specific T Cell Responses Mediated by CD4+ Cells

To examine if various pSCA1 suicidal DNA vaccines stimulated E7-specific $CD4^+$ T cell precursors to produce cytokines, double staining flow cytometry for surface CD4 and intracellular IFN-γ or IL-4 was performed to enumerate CD4+ cytokine secreting cells in splenocytes from vaccinated mice. Figures 13A and 13B show no significant difference in the number of E7-specific IFN-γ-secreting (or IL-

4-secreting) $CD4^+$ cells among the various groups. Thus, linkage of Hsp70 to E7 in a suicidal DNA vaccine did not lead to stimulation of E7-specific $CD4^+$ T cell precursors *in vivo*.

Vaccination with pSCA1-E7/Hsp70 Suicidal DNA Vaccine Did Not Induce Antti-E7 Antibodies

The quantity of anti- E7 antibodies in the sera of the vaccinated mice was determined y direct ELISA two weeks after vaccination. Sera of the mice vaccinated with pSCA1-E7/Hsp70 did not have higher titers of E7-specific antibodies compared to those mice vaccinated with pSCA1-E7 vaccine (Figure 14).

Vaccination with pSCA1-E7/Hsp70 Suicidal DNA Vaccine Protects Mice Better Against the Growth of E7-Expressing TC-1 Tumors

An *in vivo* tumor protection experiments was performed using DNA-based self-replicating pSCA1-E7/Hsp70 DNA vaccine and an E7-expressing tumor, TC-1, in C57BL/6 mice. As shown in **Figure 15**, 80% of mice receiving this vaccine remained tumor-free 70 days after TC-1 challenge. In contrast, all mice receiving wild-type pSCA1-E7 and pSCA1-Hsp70 constructs as well as all naïve mice developed tumors within 2 weeks. Therefore, the DNA-based self-replicating pSCA1-E7/Hsp70 vaccine significantly enhanced anti-tumor immunity.

Treatment with pSCA1-E7/Hsp70 Suicidal DNA Vaccine Eradicates Established Tumors in the Lungs

To determine the therapeutic potential of the DNA-based self-replicating pSCA1-E7/Hsp70 vaccine for lung metastases, each mouse was challenged with 10^4 TC-1 tumor cells i.v. Results are shown in Figure B/6A as the number of pulmonary metastatic tumor nodules ±SEM. Mice treated with the pSCA1-E7/Hsp70 suicidal DNA vaccine had the lowest number of pulmonary nodules (1.8±0.5) compared to mice vaccinated with wild-type pSCA1-E7 (47.7±4.6), pSCA1-Hsp70 (58.3±1.8), pSCA1 alone (69.0±4.9) or naïve mice (129.5±4.0) (ANOVA, $p<0.001$). Representative photographs of the lung tumors (unmagnified) are shown in Figure 16. These results indicate that the linkage of Hsp70 to E7 in a suicidal DNA vaccine significantly enhanced the antitumor therapeutic effect.

$CD8^+$ T Cells are Essential for Antitumor Effects

To determine the types of lymphocytes required for protection against E7-expressing tumors, *in vivo* antibody depletion experiments were done. As shown in Figure 17, tumors grew out within 10 days of inoculation in all naïve mice and all mice depleted of $CD8^+$ T cells. In contrast, 80% of the non-depleted mice remained tumor free 40 days after tumor challenge. Tumor grew within two weeks in 40% of mice depleted of CD4+ or of NK1.1+ cells.. These results suggest that $CD8^+$ T cells are essential for E7-specific anti-tumor immunity induced by the pSCA1-E7/Hsp70 suicidal DNA vaccine. CD4+ and NK1.1+ contributed to a lesser degree to the total antitumor effect.

DISCUSSION

The inventors have demonstrated that linkage of Hsp70 to E7 significantly enhanced the potency of an E7-expressing DNA that was administered as a suicidal DNA vaccine based on a replicating RNA replicon. Such suicidal DNA incorporating Hsp70 fused to E7 generated potent E7-specific $CD8^+$ T cell-mediated immunity. Furthermore, this chimeric pSCA1-E7/Hsp70 suicidal DNA vaccine successfully prevented lethal pulmonary metastases in an experimental metastasis model.

Stimulation of $CD8^+$ T cell activity is important in antitumor immune responses. (For review, see Chen, CH *et al., J Biomed Sci* 1998, 5:231-52; Pardoll, DM *Nat Med* 1998, 4:525-31). Such immunity was augmented by administration of a pSCA1-E7/Hsp70 suicidal DNA vaccine and was manifest as protection against tumor growth and as therapy of a pre-existing tumor. The importance of this T cell subset is emphasized by the fact that depletion of $CD8^+$ CTLs abolished this effect. Activated CTL function as effector cells that kill tumor cells directly or through the release of cytokines that interfere with tumor cell growth or survival. Therefore, the enhanced antigen-specific antitumor $CD8^+$ T cell activity is critical to the potency of the pSCA1-E7/Hsp70 vaccine.

One mechanisms by which E7-specific $CD8^+$ T cell responses are stimulated *in vivo* is the direct MHC class I-restricted presentation of E7 to $CD8^+$ T cells by APCs that express E7/Hsp70. This is known as "direct priming". However, because the suicidal DNA vaccine eventually results in the apoptosis of the very cells it transfects, direct priming by directly transfected APCs is unlikely to be effective.

Rather the enhanced $CD8^+$ T cell responses observed in pSCA1-E7/Hsp70-vaccinated mice is likely a result of "cross priming," (Huang, AY *et al., Science* 1994; 264:961-5) whereby cells expressing the E7/Hsp70 vaccine DNA release the antigen as an exogenous protein that is subsequently taken up and processed by other APCs via the MHC class I-restricted pathway. Cross-priming is the most likely mechanism for the enhanced CD8+ T cell activity because the suicidal DNA composition lyses transfected cells (Frolov *et al., Proc Natl Acad Sci U S A* 1996; 93:11371-7) leading to release of antigen which becomes available to other APCs. Previous studies reported that Hsp70 linked to malaria peptide (NANP)40 (Barrios, C *et al., Clin Exp Immunol* 1994; 98:229-33), HIV-1 p24 (Suzue *et al., supra*), ovalbumin (Suzue, K *et al., Proc Natl Acad Sci U S A* 1997; 94:13146-51), or influenza nucleoprotein (Anthony *et al., supra*) and administered as an exogenous protein enhanced MHC class I presentation of the linked antigens. Hsp70 fusion proteins are likely taken up by professional APCs which are known to be important in presenting exogenous Hsp70-associated antigens through the MHC class I pathway (Mitchell, DA *et al., Eur J Immunol* 1998; 28:1923-33; Suto, R *et al., Science* 1995; 269:1585-8). It was suggested that Hsp70 complexes can enter professional APCs via receptor-mediated endocytosis (Arnold-Schild *et al.,supra*). *Mtb* HSP protein fused to antigen stimulated DCs *in vitro* and *in vivo* to

upregulate the level of MHC class I, MHC class II and co-stimulatory molecules (Cho *et al., supra*). According to the present invention, the lytic effect of the pSCA1 vector, the enhancement of MHC class I processing, the maturation of DCs, all of which are induced by Hsp70 fused to the antigen, all contribute to augmentation of $CD8^+$ T cell activity resulting from he pSCA1-E7/Hsp70 vaccine via a cross-priming pathway.

Although use of suicidal DNA vectors and their induction of apoptosis alleviate some concerns about DNA vaccine integration into the host genome, the potency of such vaccines may be limited because of that same apoptotic outcome. The present inventors and their colleagues previously demonstrated that linkage of *Mtb* Hsp70 to E7 antigen enhanced the potency of a conventional naked DNA vaccine. (Chen *et al.*, 2000, *supra*). Here, the inventors have successfully extended the chimeric Hsp70 strategy to a suicidal DNA vector. However, the DNA-based RNA replicon vector approach appeared to be less efficacious than the conventional DNA vector approach in generating E7-specific CD8+ T cells. For example, approximately 130 E7-specific CD8+ T cells were generated per 10^6 splenocytes when vaccinating mice with the pSCA1-E7/Hsp70 composition. Meanwhile, the previous study of chimeric E7/Hsp70 in a conventional mammalian expression plasmid (pcDNA3) generated about 430 E7-specific CD8+ T cells per 10^6 splenocytes in vaccinated mice. (Chen *et al., supra*). Although DNA-based replicons may be expected produce more E7 than do conventional DNA plasmids because of their self-replicating nature, Leitner *et al.*(*supra*) showed that replicon-based DNA plasmids did not produce more antigen. Furthermore, the apoptotic outcome of transfection with a DNA-based replicon may limit direct presentation of antigen by transfected APCs to CD8+ T cells, also contributing to lower vaccine potency.

This induction of apoptosis also raises concerns about potential tissue damage of the administration of such a vaccine. However, here, microscopic examination of the vital organs of E7/Hsp70-vaccinated mice did not reveal any significant histopathological changes.

Another risk is the presence of E7 protein in host cells (since E7 is a viral oncoprotein that disrupts cell cycle regulation by binding to tumor suppressor pRB protein in nuclei) leading to potential incidence and accumulation of genetic aberrations and eventual malignant transformation. Use of the suicidal DNA vector eases the concern about oncogenicity of E7 protein since the transfected cells eventually undergo apoptosis. Oncogenicity of E7 can be further reduced by introducing mutations into E7 DNA that eliminate binding of the E7 protein to pRB (Heck, DV *et al., Proc Natl Acad Sci USA* 1992; 89:4442-6) while the cells still maintain most of their antigenicity.

In summary, the results revealed that fusion of DNA encoding *Mtb* Hsp70 to DNA encoding HPV-16 E7 in a suicidal DNA vaccine resulted in a vaccine that induced marked antigen (E7)-specific

$CD8^+$ T cell-responses manifest as a state of anti-tumor immunity against tumors expressing the antigen. Since a majority of cervical cancers express HPV E7, the present is useful for controlling of HPV-associated tumors. These findings are applicable to other tumors and types of cancer where tumor-specific antigens can be identified. Further, these findings are directly applicable to vaccines against organisms responsible for infectious diseases such as viruses, protozoa, fungi and bacteria. Because the DNA-based RNA replicon vaccines are stable and easy to prepare in mass quantities, such vaccines are particularly desirable in developing countries which have high prevalence of HPV-associated cervical malignancy while lacking facilities for storing biological agents

EXAMPLE III

Enhancement of DNA Vaccine Potency by Linking DNA Encoding Antigen to DNA Encoding the Extracellular Domain of Flt3-Ligand

Prior to the present invention, To date, FL had not been used as part of a chimeric DNA vaccine. The present inventors and their colleagues investigated whether linking a full-length E7 DNA to DNA encoding the ECD of FL would enhance the potency of a DNA vaccine. They chose HPV-16 E7 as a model antigen for vaccine development (see above).

Studies were done to compare DNA vaccines containing wild-type E7 with DNA vaccines containing full-length E7 fused to FL for their stimulation of immune responses and their ability to protect animals against growth or metastasis of E7-expressing tumors (Lin *et al.*, *supra*). The results presented below indicate that linking DNA encoding the ECD of FL to E7 dramatically increased the expansion and activation of E7-specific $CD8^+$ T cells, completely bypassing the CD4 arm. This strategy led not only to enhanced E7-specific $CD8^+$ T cell responses, but also to potent anti-tumor immunity against established metastatic tumors expressing E7.

MATERIALS AND METHODS

Plasmid DNA Constructs and Preparation

pcDNA3 was used as an expression vector (instead of a previously described pCMV-Neo-Bam vector (Chen *et al., supra*). The pcDNA3 expression vector was selected since it was used effectively to investigate the correlation between the E7-specifc T cell responses with the antitumor effects produced various DNA vaccines. The production of HPV-16 E7-expressing plasmid, pcDNA3-E7 has been described previously (Chen *et al., supra*).

For making the plasmid encoding the ECD of mouse FL, pcDNA3-FL, the DNA fragment encoding the signal peptide and ECD of mouse FL was first amplified with PCR using conditions described previously (Chen, CH *et al., Cancer Research. 60*:1035-1042., 2000) with a mouse FL DNA

template, sfHAV-EO410 (ATCC, Manassas, VA) and a set of primers: 5'-gggtctagaatgacagtgctggcgccagc-3' [SEQ ID NO:28] and 5'-gggggatccctgcctgggccgaggctctgg-3' [SEQ ID NO:29]. The amplified product was digested with XbaI and BamHI and further cloned into the XbaI and BamHI cloning sites of pcDNA3 vector (Invitrogen, Carlsbad, CA). For making pcDNA3-FL-E7, the E7 DNA fragment was isolated from pcDNA3-E7 by digestion with BamHI and HindIII and gel-recovered. The isolated fragment was cloned into the BamHI and HindIII cloning sites of pcDNA3-FL. For making pcDNA3-GFP, a DNA fragment encoding the green fluorescent protein (GFP) was first amplified in PCR using pEGFPN1 DNA (Clontech, Palo Alto, CA) and a set of primers: 5'-atcggatccatggtgagcaagggcgaggag-3' [SEQ ID NO:30] and 5'gggaagctttacttgtacagctcgtccatg-3' [SEQ ID NO:31]. The amplified product was digested with BamHI and HindIII and cloned into the BamHI and HindIII cloning sites of pcDNA3 (Invitrogen). To make pDNA3-E7-GFP, the DNA fragment encoding E7 first amplified with PCR using pcDNA3-E7 as template and a set of primers: 5'-ggggaattcatgcatggagatacaccta-3' [SEQ ID NO:32]and 5'-ggtggatccttgagaacagatgg-3' [SEQ ID NO:33]. The amplified product was digested with EcoRI and BamHI and cloned into the EcoRI and BamHI cloning sites of pcDNA3-GFP. For making pcDNA3-FL-E7-GFP, the DNA encoding the signal peptide and ECD of FL was amplified with PCR using pcDNA3-FL as a DNA template and a set of primers: 5'-gggtctagaatgacagtgctggcgccagc-3' [SEQ ID NO:34] and 5'-cgagaattcctgcctgggccgaggctctg-3' [SEQ ID NO:35]. The amplified product was digested with XbaI and EcoRI and cloned into the XbaI and EcoRI cloning sites of pcDNA3-E7-GFP vector. The accuracy of these constructs was confirmed by DNA sequencing. pcDNA3 DNA with FL, E7, FL-E7, E7-GFP or FL-E7-GFP inserts and the "empty" plasmid, pcDNA3 were transfected into subcloning-efficient DH5α™ cells (Life Technologies, USA). The DNA was then amplified and purified (Chen *et al., supra*). The integrity of plasmid DNA and the absence of *E. coli* DNA or RNA was checked in each preparation using 1% agarose gel electrophoresis. DNA concentration was determined by the optical density measured at 260 nm. The presence of inserted E7 fragment was confirmed by restriction enzyme digestion and gel electrophoresis.

The schematic domain structure of the Flt3-ligand protein and FL-E7 fusion peptide are shown in Figure 18A. The sequence of the FL-E7 construct, comprising the ECD of FL is shown in Figure 18B (SEQ ID NO:11 and 12). Residues 1-189 are FL-derived, residues 191-287 are E7-derived. The remaining residues (*e.g.*,, 288-302) are from the vector DNA.

Cell Lines

For description of TC-1 cells and their use, see above (and Lin *et al., supra*). A human embryonic kidney cell line,, 293, expressing MHC genes H-2D^b and H-2K^b (293 D^{b}K^b) (Bloom, MB *et al., J Exp Med. 185*: 453-9, 1997) was a gift from Dr. JC Yang (National Cancer Institute, NIH,

Bethesda). These cells were grown in DMEM containing 10% heat-inactivated fetal calf serum, 0.3% glutamine, 0.01 M HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 400 μg/ml G418.

The production and maintenance of TC-1 cells has been described previously (20). On the day of tumor challenge, TC-1 cells were harvested by trypsinization, washed twice with 1X Hanks buffered salt solution (HBSS) and resuspended in 1X HBSS to the designated concentration for injection. A human embryonic kidney cell line,, 293, expressing the MHC genes H-2D^b and H-2K^b (293 D^{b}K^b) (24) was a gift from Dr. JC Yang (National Cancer Institute, NIH, Bethesda, MD). These cells were grown in DMEM containing 10% heat-inactivated fetal calf serum, 0.3% glutamine, 0.01 M HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 400 μg/ml G418.

Confocal Fluorescence Microscopy

293 D^{b}K^b cells transfected with pcDNA E7-GFP and pcDNA FL-E7-GFP DNA were cultured for 24-36 hr, then cytocentrifuged onto glass slides. Cells were fixed with 4% paraformaldehyde in 1x PBS for 30 min at room temperature, permeabilized with 1x PBS containing 0.05% saponin and 1% BSA, and then incubated with mouse anti-calnexin mAb (Stressgen Biotechnologies, Victoria, Canada) at a concentration of 1 μg/ml for 30 min at room temperature. Unbound antibodies were removed by washing three times in 1X PBS. The cells were then incubated with Cy3-conjugated F(ab')$_2$ fragment of goat anti-mouse IgG (Jackson ImmunoReseach Laboratories) at a concentration of 10μg/ml for 30 min. The slides were washed with 1x PBS containing and 1% BSA. The glass slides were mounted with anti-fading medium, Mowiol 4-88 (Calbiochem Inc. La Jolla, CA) and covered with coverslips. Slides in which primary antibody was omitted were used as negative controls. Samples were examined on a confocal laser scanning microscope.

Mice

6- to 8-week-old female C57BL/6 mice from the National Cancer Institute (Frederick, MD) were purchased and kept in the oncology animal facility of the Johns Hopkins Hospital (Baltimore, MD). All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

DNA Vaccination

Preparation of DNA-coated gold particles and gene gun particle-mediated DNA vaccination was performed using a helium-driven gene gun (Bio-rad, Hercules, CA) (see Chen *et al.*, *supra*).

Intracytoplasmic Cytokine Staining and Flow Cytometry Analysis

Pooled splenocytes from groups of naïve or vaccinated mice (5 mice per group) were incubated either with

(a) the E7 peptide (aa 49-57) including an MHC class I epitope (Fetlkamp *et al.*, *supra*) for detecting E7-specific $CD8^+$ T cell precursors,, or

(b) the E7 peptide (aa 30-67) containing the MHC class II peptide (Tindle *et al.*, *supra*) for detecting E7-specific $CD4^+$ T helper cell precursors.

E7 peptide was added at 2μg/ml for 20 hours. Golgistop (Pharmingen, San Diego, CA) was added 6 hours before harvesting cells from the culture. Cells were washed once in FACScan buffer and stained with phycoerythrin (PE)-conjugated monoclonal rat anti-mouse CD8 or CD4 antibody (PharMingen, San Diego, CA). Cells were subjected to intracellular cytokine staining using the Cytofix/Cytoperm kit according to the manufacturer's instructions (PharMingen). FITC-conjugated anti-IFN-γ antibody and the immunoglobulin isotype control antibody (rat IgG1) were all purchased from PharMingen. Analysis was done on a Becton Dickinson FACScan with CELLQuest software (Becton Dickinson Immunocytometry System, Mountain View, CA).

ELISA for anti-E7 antibody (see EXAMPLE I)

In vivo Tumor Protection

These studies were generally as Example I and II: gene gun with 2 μg of FL DNA, E7 DNA, FL-E7 DNA, FL mixed with E7 (FL+E7), or unvaccinated. One week later, mice were boosted with the same regimen and challenged subcutaneously on day 14 with 10^4 TC-1 tumor cells in the right leg. Statistical analysis was performed using SAS version 6.12 (SAS Institute Inc., Cary, NC, USA). Percent of tumor free mice was analyzed using the Kaplan-Meier analysis. Statistical significance was tested using log-rank statistics.

Tumor Therapy

The tumor cells and DNA vaccines were prepared as above. See Example II for description of tumor challenge. Three days later, mice were given 2 μg of a vaccine preparation ((FL DNA, E7 DNA, FL-E7 DNA via gene gun or were left unvaccinated)). One week later, animals were boosted using the same regimen and were sacrificed on day 25. Lung tumor nodules were evaluated as in Example II. Statistical significance was tested using one-way ANOVA.

In vivo Antibody Depletion Experiments

The procedure was done as in Example II

Generation of DCs

DCs were generated by culturing bone marrow cells in the presence of GM-CSF as described previously (Fernanadez, NC *et al.*, *Nat Med.* 5: 405-11, 1999). Briefly, bone marrow was collected from mouse femurs and tibias. Erythrocytes were lysed, and the remaining cells were passed through a

nylon mesh to remove small pieces of bone and debris. The cells were collected and 10^6 cells /ml were placed in 24-well plates in RPMI 1640 medium supplemented with 5% FCS, 2mM β-mercaptoethanol, 1% nonessential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Rockville, MD) and 100 U/ml GM-CSF (PharMingen, San Diego, CA). Two-thirds of the medium was replaced every 2 days. Non-adherent cells were harvested on day 7 and characterized by flow cytometry for DC markers as previously described (Wang, TL *et al., Gene Therapy. 7*: 726-733., 2000).

Generation of E7-Specific $CD8^+$ T Cell Lines

See Wang *et al., supra*. Briefly, female C57BL/6 (H-2^b) mice were immunized intraperitoneally with vaccinia-Sig/E7/LAMP-1. Splenocytes were harvested on day 8. The cells were incubated with IL-2 (20 U/ml) and E7 peptide (aa 49-57) (1 μM) for 6 days. Cells of the E7-specific CTL cell line were propogated in 24-well plates by mixing, in a final volume of 2 ml, (a) 10^6 splenocytes that included the E7-specific CTLs (b) 3 x 10^6 irradiated splenocytes (c) IL-2 (20 U/ml) and (d) E7 peptide (aa 49-57) at 1 μM. This procedure was repeated every 6 days. The target-cell specificity of the E7 CTL line was characterized in a CTL assay. Flow cytometry was used demonstrate CD8 expression.

CTL Assay using Transfected 293 D^bK^b Cells as Target Cells

CTL assays were performed in 96-well round-bottom plates as described by Corr *et al.,* (Corr, M *et al., J Immunol. 163:* 4721-7, 1999) and in Examples I and II. Transfected 293 D^bK^b cells were used as target cells while E7-specific $CD8^+$ T cells served as effectors. 5x10^6 293 D^bK^b cells were transfected with 20 μg of pcDNA3 (empty plasmid), E7, FL, or FL-E7 DNA vaccines with lipofectamine 2000 (Life Technologies, Rockville, MD) according to manufacturer's instructions. Cells were collected 40-44 hr after transfection. Levels of E7 protein expression , determined by ELISA, were similar in E7 and FL-E7-transfected cells. Cells were incubated and lysis measured as above.

CTL Assay Using DCs Pulsed with Lysates of Transfected 293 D^bK^b Cells as Target Cells

CTL assays using as targets DCs pulsed with cell lysates were generally in accordance with Uger, RA *et al., J Immunol. 160*: 1598-605, 1998. Briefly, 293 D^bK^b cells were transfected as above and subjected to three freeze-thaw cycles. Protein concentrations were determined using the BioRad protein assay (Bio-Rad, Hercules, CA) using the vendor's protocol. The quantity of E7 protein was determined by ELISA. Cell lysates from E7 or FL-E7 DNA transfected 293 D^bK^b cells were standardized for E7 protein concentration.

DCs were prepared for use as target cells by pulsing 10^6 DCs with different concentrations of cell lysates (50, 10, 2 and 0.4 μg/ml) in a final volume of 2 ml for 16-20 hrs. E7-specific $CD8^+$ T cells were effector cells. CTL assays were performed at a fixed E/T ratio of 9 using 9x10^4 T cells and 10^4

prepared DC targets in a final volume of 200 µl. Results were determined by measurements of LDH as above.

RESULTS

Linkage of the Extracellular Domain of FL to E7 Protein Re-routes E7 into the Endoplasmic Reticulum

To determine the expression and localization of wild-type E7 and E7 fusion proteins, DNA encoding the green fluorescent protein (GFP) was linked to the 3' end of E7 DNA and chimeric FL-E7 DNA as a tag. Transfection and subsequent examination by fluorescence microscopy was used to determine the expression and localization of wild-type and modified E7 protein. Levels of protein expression was quite similar between cells transfected with E7-GFP or FL-E7-GFP. As expected, cells transfected with the E7-GFP showed cytoplasmic/ nuclear distribution. In comparison, cells transfected with the chimeric FL-E7-GFP displayed a network pattern consistent with endoplasmic reticulum (ER) localization. To test whether the FL-E7-GFP chimera had in fact been distributed to the ER, cells were further stained with an antibody to calnexin and examined by immunofluorescence. Calnexin is a well-characterized marker for the ER. Co-localization of E7-GFP and calnexin was only observed in cells transfected with FL-E7-GFP but not E7-GFP, indicating that at least some of the FL-E7 fusion product but not E7-GFP was targeted to ER compartments. These results indicated that the addition of the ECD of FL to E7 facilitates the entry into ER compartments.

Vaccination with FL-E7 Fusion DNA Significantly Enhanced E7-Specific $CD8^+$ T Cell Responses

$CD8^+$ T lymphocytes are important effectors of anti-tumor immunity. As a measure of the E7-specific $CD8^+$ T cell response generated by the FL-E7 DNA vaccine, intracellular IFNγ cytokine staining was evaluated in splenocytes from vaccinated mice. This is a sensitive functional assay for measuring IFN-γ production at the single-cell level (Murali-Kristna, K *et al., Immunity. 8*: 177-87).

As shown in Figure 19A and B, vaccination of mice with FL-E7 DNA generated the highest number of E7-specific IFN-γ^+ $CD8^+$ T cell precursors (940 per $3x10^5$ splenocytes) compared to vaccination with E7 DNA (12 per $3x10^5$ splenocytes) ($p<0.01$). Thus, FL-E7 DNA immunization led to a nearly 8-fold increase in the number of E7-specific $CD8^+$ T cell precursors. These results also indicated that fusion of E7 to FL was required for this enhancement because vaccination with a mixture of FL-DNA and E7-DNA did not generate such enhanced $CD8^+$ T cell activity.

Vaccination with FL-E7 Fusion DNA Did not Induce Significant E7-Specific CD4+ T Cell Responses or Anti-E7 Antibodies

E7-specific $CD4^+$ T precursor cells secreting the cytokine IFN-γ or IL-4) were assessed by double staining for surface CD4 and intracellular IFN-γ or IL-4 using flow cytometry Splenocytes were from immunized mice. Figure 20A shows that mice vaccinated with FL-E7 DNA developed no significant increase in

$CD4^+$ IFN-γ^+ double positive cells compared to mice vaccinated with FL DNA, wild-type E7 DNA, plasmid DNA or unvaccinated naïve mice. Positive control splenocytes were from Sig/E7/LAMP-1 DNA vaccinated mice (Ji *et al.*, *supra*). Similarly, no significant increases in $CD4^+$ IL-4^+ double-positive cells were observed (FL-E7 vaccinated compared with FL DNA, wild-type E7 DNA, plasmid DNA or unvaccinated naïve mice (**Figure 20B**). IL-4-secreting activated mouse splenocytes (MiCK-2, PharMingen) were positive controls to assure successful intracellular IL-4 staining.

To determine the levels of E7-specific antibodies in the sera of the vaccinated mice, ELISA was performed 2 weeks after the last vaccination. No significant E7-specific antibody responses were detected in mice. Sera of the mice vaccinated with chimeric FL-E7 DNA did not have higher titers of E7-specific antibodies compared to mice vaccinated with FL, empty plasmids, or unvaccinated naïve mice.

Vaccination with Chimeric FL-E7 DNA Vaccine Protects Mice Better Against the Growth of E7-Expressing TC-1 Tumors

Results of an *in vivo* tumor protection study is shown in Figure 21. 100% of mice vaccinated with FL-E7 DNA remained tumor-free 70 days after challenge (log-rank, $p<0.001$). In contrast, only 20% of mice receiving wild-type E7 remained tumor free after day 32 and all unvaccinated mice, or mice given FL DNA developed tumors within 20 days of challenge. Fusion of E7 to FL was required for generating protective immunity, since only 20% of mice vaccinated with a mixture E7 DNA and FL DNA) remained tumor free after 32 days. Therefore, FL-E7 fusion DNA significantly enhanced anti-tumor immunity.

Treatment with FL-E7 Fusion DNA Eradicates Established E7-expressing Tumors in the Lungs

To determine the therapeutic potential of a chimeric FL-E7 DNA construct in treating TC-1 lung metastases, each mouse was challenged with tumor cells i.v. Results are shown in **Figure 22A** as the mean number of pulmonary metastatic tumor nodules ±SEM. Mice vaccinated with FL-E7 DNA had the lowest mean number of pulmonary nodules (5.8±3.6) compared to mice vaccinated with wild-type E7 DNA (67.5±3.5), FL DNA mixed with E7 DNA (68±15), FL DNA (65.0±5.0) or unvaccinated mice (50.7±7.3) (one-way ANOVA, $p<0.001$). **Figure 22B** shows lung weights (mean ±SEM in grams). Mice vaccinated with FL-E7 DNA had the lowest lung weight (0.158±0.025) compared to mice vaccinated with wild-type E7 DNA (0.462±0.02), FL DNA plus E7 DNA (0.469±0.08), or FL DNA (0.6±0.03), or unvaccinated mice (0.645±0.08) (one-way ANOVA, $p<0.001$). Representative photographs of the lung tumors are shown in **Figure 23**.

$CD8^+$ T Cells But Not $CD4^+$ T cells are Essential for Anti-tumor Effects

To determine the types of lymphocytes required for protection against E7-expressing tumors, *in vivo* antibody depletion experiments (Lin *et al.*, *supra*; Wu *et al.*, 1995, *supra*) were done. Depletion of lymphocyte subsets was assessed on the day of tumor injection, and weekly thereafter by flow cytometric analysis of spleen cells. More than 99% of cells of the appropriate subset were depleted in all cases without effect on the other subsets. As shown in **Figure 24**, 100% of unvaccinated mice and mice depleted of $CD8^+$ T cells grew tumors within 14 days after challenge. In contrast, all non-depleted mice and all mice depleted of $CD4^+$ T cells remained tumor-free 60 days after challenge. 40% of mice in which NK1.1+ cells were depleted grew tumors 6 weeks after challenge. Even though there appeared to have been an effect of NK cell depletion, the difference from controls is result was not statistically significant (log-rank, p=.13). It was concluded that $CD8^+$ T cells are essential for E7-specific antitumor immunity induced by the FL-E7 DNA vaccine.

Enhanced Presentation of E7 via the MHC Class I Pathway in Cells Transfected with FL-E7 DNA

As noted earlier, mice vaccinated with FL-E7 generated the highest number of E7-specific $CD8^+$ T cell precursors. To understand the mechanism underlying this effect, the inventors tested whether enhanced MHC class I presentation of E7 occurred in cells expressing FL-E7 (in this case, human embryonic kidney 293 cells D^bK^b transfected with FL-E7). CTL assays employing D^b-restricted E7-specific $CD8^+$ effector T cells were used to determine if target 293 D^bK^b cells transfected with FL-E7 were killed more efficiently than 293 D^bK^b cells transfected with wild-type E7. 293 D^bK^b cells were selected because of their stable and high transfection efficiency (Bloom *et al.*, *supra*). In addition, levels of E7 expression in 293 D^bK^b cells transfected with FL-E7 DNA or E7 DNA were similar.

In the CTL assays, targets were 293 D^bK^b cells that had been transfected with either empty plasmid, FL DNA, E7 DNA, or FL-E7 DNA, or that were not transfected.. Effector cells were added to achieve various E/T ratios (1, 3, 9, 27). As shown in **Figure 25**, 293 D^bK^b cells transfected with FL-E7 DNA were lysed at a higher level than targets cells transfected with wild-type E7 DNA. Transfection with FL-E7 DNA thus resulted in more efficient presentation of E7 antigen via the MHC class I pathway.

Enhanced Presentation of E7 Through the MHC Class I Pathway in DCs Pulsed With Chimeric FL-E7 Protein

Enhanced E7-specific $CD8^+$ T cell responses *in vivo* may occur as a result of presentation of E7 via the MHC class I pathway resulting from uptake of lysed cellular material expressing various E7 constructs by host APCs ("cross-priming").

A cross priming experiment was performed to characterize the MHC class I presentation of E7 of DCs pulsed with cell lysates of 293 D^bK^b cells transfected with empty plasmid, FL, E7, or FL-E7 DNA.

Lysates of transfected 293 D^bK^b cells were obtained by repeated cycles of freeze-thaw. 10^6 bone marrow-derived DCs were pulsed with serial dilutions (50, 10, 2 or 0.4 μg) of lysate derived from 293 D^bK^b cells transfected with different constructs. These DCs were used as target cells for lysis by D^b-restricted E7-specific $CD8^+$ CTL. CTL assays were performed at a fixed E/T ratio of 9. As shown in **Figure 26**, DCs pulsed with lysates from 293 D^bK^b cells transfected with FL-E7 DNA were lysed at a higher percentage compared to DCs pulsed with lysates from 293 D^bK^b cells transfected with the other DNA constructs and non-transfected DCs. It was concluded that DCs pulsed with FL-E7 fusion protein presented E7 antigen through the MHC class I pathway more efficiently than DCs pulsed with wild-type E7 protein. Thus, the fusion of FL to E7 enhanced E7-specific $CD8^+$ T cell immune responses via cross priming effects.

DISCUSSION

The foregoing study demonstrated that linking the ECD of FL to E7 significantly enhanced the potency of E7-expressing DNA vaccines to induce potent $CD8^+$ T cell-immune responses that were protective and therapeutic against E7-expressing tumors, such that mice were protected from both primary tumor growth and development of lethal pulmonary metastases.

The incorporation of FL into the vaccine preferentially enhanced $CD8^+$ T cell responses vs. E7-specific $CD4^+$ T cell responses that were not significantly changed. Linking FL to E7 in the vaccine directly enhanced MHC class I presentation of E7 (compared to a wild-type E7 vaccine) in transfected cells *in vitro*. Since biolistic DNA delivery introduces DNA directly into dermal professional APCs, FL-E7 DNA-transfected APCs may act by directly presenting E7 via the MHC class I pathway to $CD8^+$ T cells *in vivo*.

Although it is not clear how this linkage directly enhances MHC class I presentation, one mechanism involves a chaperone effect of FL. When expressed in cells, FL may be distributed to the ER (Chklovoskaia, E *et al., Blood. 93*: 2595-604, 1999). Fluorescence microscopic examination revealed that in cells transfected with FL-E7-GFP, most of the FL-E7-GFP fusion protein co-localized with calnexin in the ER, suggesting that this linkage facilitates entry of E7 into the ER. Several studies demonstrated that ER targeting can enhance antigen-specific MHC class I-restricted CTL activity (Shiver, JW *et al., J Pharm Sci 85*: 1317-24, 1996; Hsu, SC *et al., Int Immunol. 10*: 1441-7, 1998).

Another mechanism that may contribute to the present observations is "cross-priming," whereby lysis of cells expressing FL-E7 releases protein that is taken up and processed by other APCs via the

MHC class I-restricted pathway. The present results show that DCs pulsed with FL-E7 fusion protein are capable of presenting E7 antigen through the MHC class I pathway in a more efficient manner than DCs pulsed with wild-type E7 protein. (Figure 26). However, the "cross-priming" of chimeric FL-E7 probably does not play a major role in gene gun-mediated FL-E7 DNA vaccination. Direct priming, but not cross-priming, of $CD8^+$ T cells by DNA-transfected DCs is the key event in gene gun-mediated DNA immunization (Porgador, A *et al., J Exp Med. 188:* 1075-82, 1998; Akbari, O *et al., J Exp Med. 189*: 169-78, 1999). However, the possibility of cross-priming cannot be ruled out, because FL-E7 might be released from other cell types, such as keratinocytes (which are also transfected by gene gun vaccination), and then enter DCs via a cross-priming mechanism.

No significant increases in the numbers of DCs or NK cells were detected in the spleens of mice vaccinated with FL-E7 DNA vaccines even though FL is known to expand these cell populations (Peron *et al., supra*; Williams, NS *et al., J Exp Med. 186:* 1609-14, 1997, Shaw, SG *et al., J Immunol. 161:* 2817-24, 1998). This may be related to small amounts of FL-E7 present in the circulation after DNA vaccination. FL-E7 protein could not be detected in sera of mice vaccinated with FL-E7 DNA, which also raises a question about the source of FL-E7 protein for cross-priming. FL-E7 protein from the lysis of transfected keratinocytes may be taken up by Langerhans' cells and further processed in the draining lymph nodes without entering the circulation in detectable quantities.

The E7 DNA vaccine described above had weaker antitumor effects compared to an E7 DNA vaccine using a different mammalian expression vector (Chen *et al.,* 1999, *supra* Ji *et al., supra*). In these previous studies, a pCMV-Neo-Bam expression vector that includes the HCMV promoter was used E7 DNA vaccine using that vector generated a very impressive antitumor effect in the relative absence of E7-specific $CD8^+$ T cell responses. In the current study, a relatively weak E7-specific $CD8^+$ T cell response and a relatively weak anti-tumor response were observed in mice vaccinated with E7 DNA in the form of the pcDNA3 vector. The discrepancy in the anti-tumor response evoked by the same DNA in a different vector may be explained simply by different levels of expression. Furthermore, bacterial DNA can contain immunostimulatory elements such as CpG islands (Sato, Y *et al., Science. 273:* 352-4, 1996; Klinman, DM *et al., J Immunol. 158:* 3635-9, 1997), which can cause simultaneous maturation and activation of DCs (Sparwasser, T *et al., Eur J Immunol. 28:* 2045-54, 1998) thereby acting as an adjuvant for tumor immunization (Weiner, GJ *et al., Proc Natl Acad Sci USA. 94:* 10833-7, 1997).

The FL-E7 DNA vaccine may raise certain safety concerns because DNA could integrate into the host genome, though it is estimated that the frequency of integration is much lower than that of spontaneous mutation and should not pose any real risk (Nichols, WW *et al., Annals of NY Academy of Science. 772:* 30-39., 1995). The risks of HPV-16 E7 protein was discussed above. There is a concern about possible autoimmune effects resulting from excessive expansion of DCs *in vivo*. However, here,

no significant increase in the numbers of DCs was observed in the spleen and lymph nodes of mice vaccinated with FL or FL-E7 DNA vaccines. Examination of vital organs in all of the FL-E7-vaccinated mice did not reveal any significant gross or microscopic pathology. Therefore, FL-E7 can be used as a potent DNA vaccine without observable detrimental side effects.

In summary, fusion of DNA encoding the ECD of the FL protein to E7 DNA generated potent E7-specific $CD8^+$ T cell responses and anti-tumor effects against E7-expressing tumors. Linkage of DNA encoding FL to DNA encoding an antigen enhances the potency of DNA vaccines and are applicable to other tumors and types of cancer where tumor-specific antigens can be identified. Further, these findings are directly applicable to vaccines against organisms responsible for infectious diseases such as viruses, protozoa, fungi and bacteria.

The references cited above are all incorporated by reference herein, whether specifically incorporated or not. All publications, patents, patent applications, GenBank sequences and ATCC deposits, cited herein are hereby expressly incorporated by reference for all purposes.

Citation of the documents herein is not intended as an admission that any of them is pertinent prior art. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicant and does not constitute any admission as to the correctness of the dates or contents of these documents.

WHAT IS CLAIMED IS:

1. A nucleic acid molecule encoding a fusion polypeptide useful as a vaccine composition, which molecule comprises:

(a) a first nucleic acid sequence encoding a first polypeptide or peptide that (i) promotes processing via the MHC class I pathway (MHC-I-PP) and/or (ii) promotes development or activity of an antigen presenting cell (APC);

(b) optionally, fused in frame with the first nucleic acid sequence, a linker nucleic acid sequence encoding a linker peptide; and

(c) a second nucleic acid sequence that is linked in frame to said first nucleic acid sequence or to said linker nucleic acid sequence and that encodes an antigenic polypeptide or peptide.

2. The nucleic acid molecule of claim 1, wherein the antigenic peptide comprises an epitope that binds to a MHC class I protein.

3. The nucleic acid molecule of claim 2, wherein said epitope is between about 8 amino acid residues and about 11 amino acid residues in length.

4. The nucleic acid molecule of any of claims 1-3 wherein the first polypeptide or peptide is Hsp70, an active C-terminal domain thereof, or a functional derivative of Hsp70 or of said C-terminal domain.

5. The nucleic acid molecule of any of claims 1-3 wherein the first polypeptide or peptide is the Flt3 ligand (FL), the extacellular domain thereof, or or a functional derivative of FL or of said extracellular domain.

6. The nucleic acid molecule of claim 4, wherein the first polypeptide is SEQ ID NO:4 or the full length sequence of Hsp70 a set forth in GENBANK Z95324 AL123456 and encoded by nucleotides 10633-12510 of the *Mycobacterium tuberculosis* genome.

7. The nucleic acid molecule of claim 4 wherein the first polypeptide consists essentially of said C-terminal domain having a sequence from about residue 517 to about the C-terminal amino acid residue of:

(i) SEQ ID NO:4, or

(ii) the full length native sequence of Hsp70 a set forth in GENBANK Z95324 AL123456 and encoded by nucleotides 10633-12510 of the *Mycobacterium tuberculosis* genome.

8. The nucleic acid molecule of claim 5, wherein the first polypeptide is FL.

9. The nucleic acid molecule of claim 5, wherein the first polypeptide polypeptide consists essentially of the sequence SEQ ID NO:10.

10. The nucleic acid molecule of any of claims 1-9 wherein the antigen is one which is present on, or/ cross-reactive with an epitope of, a pathogenic organism, cell, or virus.

11. The nucleic acid molecule of claim 10, wherein the virus is a human papilloma virus.

12. The nucleic acid molecule of claim 11, wherein the antigen is the E7 polypeptide of HPV-16 or an antigenic fragment thereof.

13. The nucleic acid molecule of claim 12, wherein the HPV-16 E7 polypeptide is non-oncogenic.

14. The nucleic acid molecule of claim 10, wherein the pathogenic organism is a bacterium.

15. The nucleic acid molecule of claim 10, wherein the pathogenic cell is a tumor cell.

16. The nucleic acid molecule of claim 15, wherein the antigen is a tumor-specific or tumor-associated antigen, or any antigenic epitope thereof.

17. The nucleic acid molecule of claim 16, wherein the antigen comprises the HER-2/neu protein or a peptide thereof, mutant p53 or a melanoma-associated antigens selected from the group consistingof MAGE-1, MAGE-3, MART-1/Melan-A, tyrosinase, gp75, gp100, BAGE, GAGE-1, GAGE-2, GnT-V, and p15.

18. The nucleic acid molecule of any of claims 1-17 operatively linked to a promoter.

19. The nucleic acid molecule of claim 18 , wherein the promoter is one which is expressed in an APC.

20. The nucleic acid molecule of claim 21, wherein the APC is a dendritic cell.

21. An isolated nucleic acid molecule that, under stringent hybridization conditions, hybridizes simultaneously with:

(i) at least part of said first nucleic acid sequence and at least part of said second nucleic acid sequence,

(ii) at least part of said first nucleic acid sequence and part of said linker nucleic acid sequence,

(iii) at least part of said second nucleic acid sequence and part of said linker nucleic acid sequence, or

(iv) at least part of said first nucleic acid sequence, at least part of said first nucleic acid sequence and said linker nucleic acid sequence,

said sequences in accordance with any of claims 1-20.

22. An expression vector comprising the nucleic acid molecule of any of claims 1-20 operatively linked to

(a) a promoter; and

(b) optionally, additional regulatory sequences that regulate expression of said nucleic acid in a eukaryotic cell.

23. The expression vector of claim 22 which is a viral vector or a plasmid.

24. The expression vector of claim 22 wherein said plasmid is pcDNA3 which is derived from the sequence, prior to insertion of the nucleic acid sequences encoding said first or said second polypeptides, of SEQ ID NO:15.

25. The expression vector of claim 22 which is a self-replicating RNA replicon.

26. The expression vector of claim 25, wherein the self-replicating RNA replicon is a Sindbis virus self-replicating RNA replicon.

27. The expression vector of any of claims 25 or 26, wherein the replicon is SINrep5 which is derived from the sequence, prior to insertion of the nucleic acid sequences encoding said first or said second polypeptides, of SEQ ID NO:16.

28. The expression vector of claim 22 which is a suicidal DNA vector.

29. The expression vector of claim 28 wherein said suicidal DNA vector is an alphavirus DNA vector.

30. The expression vector of claim 29 wherein said alphavirus is Semliki Forest virus (SFV).

31. The expression vector of claim 30 wherein said SFV vector is pSCA1.

32. The expression vector of any of claims 28-30 wherein the suicidal DNA is derived from the sequence, prior to insertion of the nucleic acid sequences encoding said first or said second polypeptides, of SEQ ID NO:17.

33. The expression vector of any of claims 22-32 wherein the first encoded polypeptide or peptide is Hsp70, an active C-terminal domain thereof, or a functional derivative of Hsp70 or of said C-terminal domain.

34. The expression vector of claim 33 wherein the first encoded polypeptide consists essentially of the sequence SEQ ID NO:4 or the full length sequence of Hsp70 a set forth in GENBANK Z95324 AL123456 and encoded by nucleotides 10633-12510 of the *Mycobacterium tuberculosis* genome.

35. The expression vector of claim 32 wherein the first polypeptide consists essentially of said C-terminal domain having a sequence from about residue 517 to about the C-terminal amino acid residue of:

(i) SEQ ID NO:4, or

(ii) the full length native sequence of Hsp70 a set forth in GENBANK Z95324 AL123456 and encoded by nucleotides 10633-12510 of the *Mycobacterium tuberculosis* genome.

36. The expression vector of an of claims 34, that is a self-replicating RNA replicon that also encodes HPV protein E7, and has the nucleotide sequence SEQ ID NO:19.

37. The expression vector of claim 34, that is a suicidal DNA vector which also encodes HPV protein E7, and has the nucleotide sequence of SEQ ID NO:20.

38. The expression vector of any of claims 22-32 wherein the first encoded polypeptide or peptide is the Flt3 ligand (FL), the extacellular domain thereof, or or a functional derivative of FL or of said extracellular domain.

39. The expression vector of claim 38 wherein the first encoded polypeptide consists essentially of the extracellular domain of FL having a sequence SEQ ID NO:10.

40. The expression vector of claim 39 that comprises a naked DNA plasmid pcDNA3 that includes the coding sequence for HPV protein E7 and the FL extracellular domain, and has the nucleotide sequence SEQ ID NO:21.

41. A cell which has been modified to comprise the nucleic acid or expression vector of any of claims 1-40.

42. The cell of claim 41 which expresses said nucleic acid molecule and said fusion polypeptide.

43. The cell of claim 41 or 42 which is an APC.

44. The cell of claim 43, wherein the APC is a dendritic cell, a keratinocyte, a macrophage, a monocyte, a B lymphocyte, a microglial cell, an astrocyte, or an activated endothelial cell.

45. A particle comprising the nucleic acid or expression vector of any of claims 1-40.

46. The particle of claim 45 which comprises a material is suitable for introduction into a cell or an animal by particle bombardment.

47. The particle of claim 46, wherein the material is gold.

48. A fusion or chimeric polypeptide comprising

(a) a first polypeptide or peptide that (i) promotes processing via the MHC class I pathway and/or (ii) promotes development or activity of an APC; and

(b) a second polypeptide comprising an antigenic peptide or polypeptide.

49. The fusion or chimeric polypeptide of claim 48, wherein the antigenic peptide or polypeptide comprises an epitope that binds to, and is presented on the cell surface by, MHC class I proteins.

50. The fusion or chimeric polypeptide of claim 48 or 49 wherein the first polypeptide and the antigenic polypeptide or peptide are linked by a chemical linker.

51. The fusion polypeptide of any of claims 48-50 wherein the first polypeptide is N-terminal to the second polypeptide.

52. The fusion polypeptide of any of claims 48-50 wherein the second polypeptide is N-terminal to the first polypeptide.

53. The fusion or chimeric polypeptide of any of claims 48-52 wherein the first polypeptide or peptide is Hsp70, an active C-terminal domain thereof, or a functional derivative of Hsp70 or of said C-terminal domain.

54. The fusion or chimeric polypeptide of any of claims 48-52 wherein the first polypeptide or peptide is the Flt3 ligand (FL), the extacellular domain thereof, or or a functional derivative of FL or of said extracellular domain.

55. The fusion polypeptide of claim 53, wherein the first polypeptide is SEQ ID NO:4 or the full length sequence of Hsp70 a set forth in GENBANK Z95324 AL123456 and encoded by nucleotides 10633-12510 of the *Mycobacterium tuberculosis* genome.

56. The fusion polypeptide of claim 8, wherein the first polypeptide polypeptide consists essentially of the sequence SEQ ID NO:10.

57. The fusion polypeptide encoded by the nucleic acid molecule of any of claims 1-20.

58. The fusion polypeptide encoded by the expression vector of any of claims 22-40.

59. A pharmaceutical composition capable of inducing or enhancing an antigen-specific immune response, comprising:

(a) pharmaceutically and immunologically acceptable excipient in combination with;

(b) a composition selected from:

(i) the nucleic acid molecule or expression vector of any of claims 1-40;

(ii) the cell of any of claims 41-44;

(iii) the particle of any of claims 45-47;

(iv) the fusion or chimeric polypeptide of any of claims 48-58; or

(v) any combination of (i)-(iv).

60. A method of inducing or enhancing an antigen specific immune response in a subject comprising administering to the subject an effective amount of the pharmaceutical composition of claims 59, thereby inducing or enhancing said response.

61. The method of claim 60, wherein the response is mediated at least in part by $CD8^+$ cytotoxic T lymphocytes (CTL).

62. The method of claim 60, wherein the response is mediated at least in part by antibodies.

63. The method of any of claims 60-62 wherein said subject is a human.

64. A method of inducing or enhancing an antigen specific immune response in cells or in a subject comprising contacting said cells with, or administering to said subject, an effective amount of the pharmaceutical composition of claim 59, thereby inducing or enhancing said response.

65. The method of claim 64, comprising contacting said cells *ex vivo* with said composition.

66. The method of claim 65 wherein said cells comprise APCs.

67. The method of claim 66, wherein said APCs are dendritic cells.

68. The method of claim 66 or 67, wherein the APCs or said dendritic cells are of human origin..

68. The method of any of claims 66-68, wherein the APCs are isolated from a living subject.

69. The method of any of claims 65-69, further comprising a step of administering said cells to which were contacted with the composition *ex vivo* to (i) a histocompatible subject or (ii) the subject from which said cells were obtained.

70. The method of any of claims 64-69 wherein said cells are human cells and said subject is a human.

71. The method of any of claims 60-64 and 69-70 wherein said administering is by a intramuscular, intradermal, or subcutaneous route.

72. The method of any of claims 60-64 and 69-70, wherein

the composition comprises said nucleic acid molecule, said expression vector or said particle, and

said administering is by biolistic injection.

73. The method of any of claims 60-64, 69-72 wherein the administering is intratumoral or peritumoral.

74. A method of increasing the numbers or lytic activity of $CD8^+$ CTLs specific for a selected antigen in a subject, comprising administering to said subject an effective amount of the pharmaceutical composition of claim 59, wherein

(i) said nucleic acid molecule, said expressio vector, said cell, said particle or said fusion or chimeric polypeptide comprises said selected antigen, and

(ii) said selected antigen comprises an epitope that binds to, and is presented on the cell surface by, MHC class I proteins,

thereby increasing the numbers or activity of said CTLs.

75. A method of inhibiting growth or preventing re-growth of a tumor in a subject, comprising administering to said subject an effective amount of a pharmaceutical composition of claim 59, wherein said nucleic acid molecule, said expression vector, said cell, said particle or said fusion or chimeric polypeptide comprises one or more tumor-associated or tumor-specific epitopes present on said tumor in said subject, thereby inhibiting said growth or preventing said re-growth.

76. The method of claim 75, wherein said administering is intratumoral or peritumoral.

77. The method of any of claims 63 or 64, further comprising treating said subject with radiotherapy or chemotherapy.

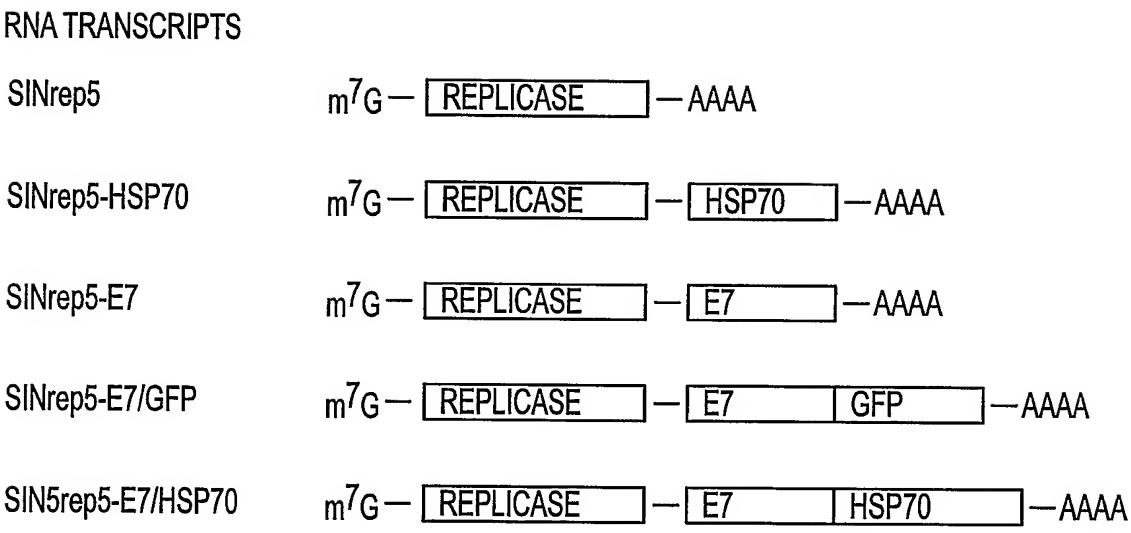

RNA TRANSCRIPTS
SINrep5
m7G — REPLICASE — AAAA
SINrep5-HSP70
m7G — REPLICASE — HSP70 — AAAA
SINrep5-E7
m7G — REPLICASE — E7 — AAAA
SINrep5-E7/GFP
m7G — REPLICASE — E7 GFP — AAAA
SIN5rep5-E7/HSP70
m7G — REPLICASE — E7 HSP70 — AAAA

FIG. 1

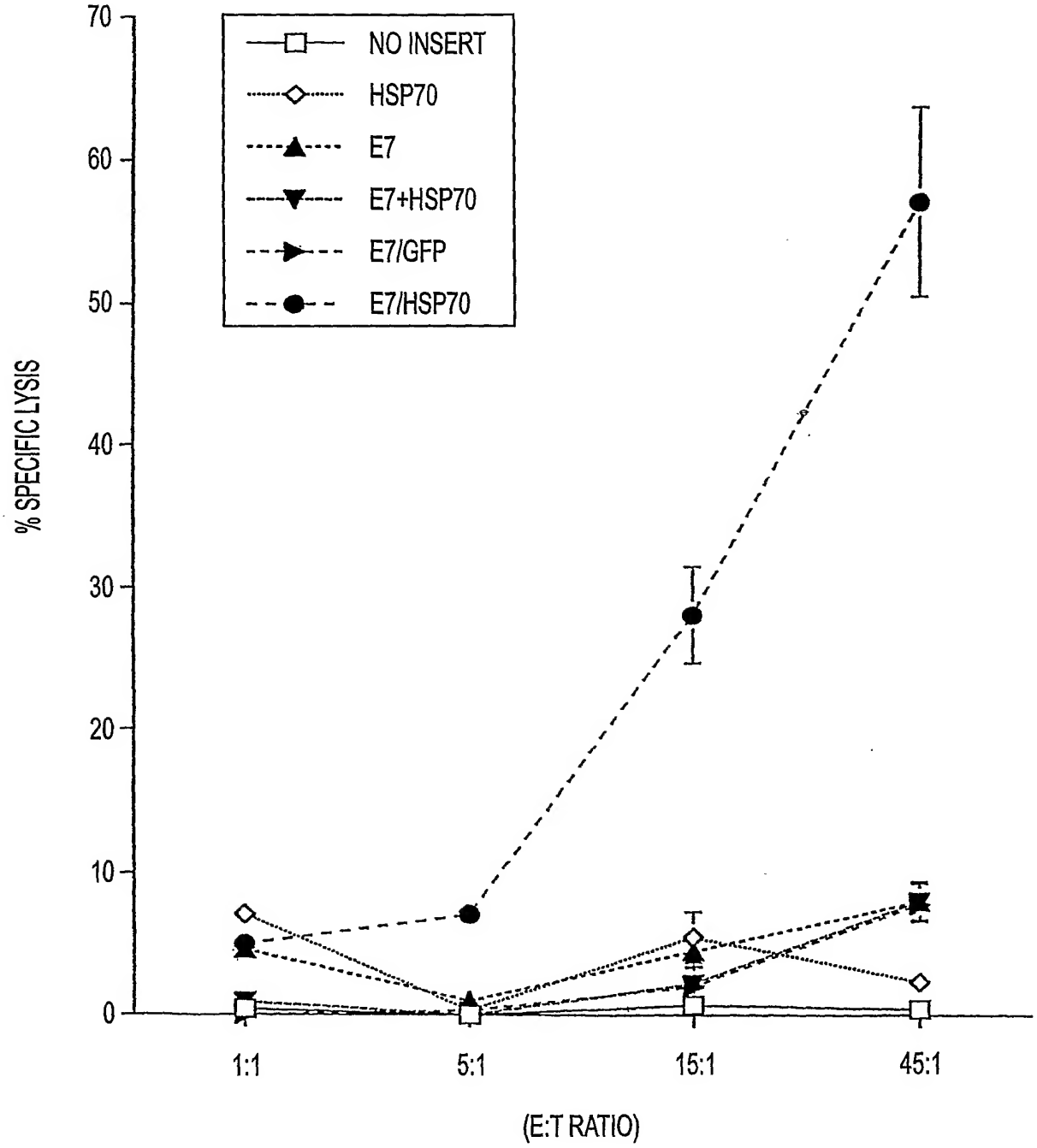

NO INSERT
HSP70
E7
E7+HSP70
E7/GFP
E7/HSP70
% SPECIFIC LYSIS
70
60
50
40
30
20
10
0
1:1
5:1
15:1
45:1
(E:T RATIO)

FIG. 2

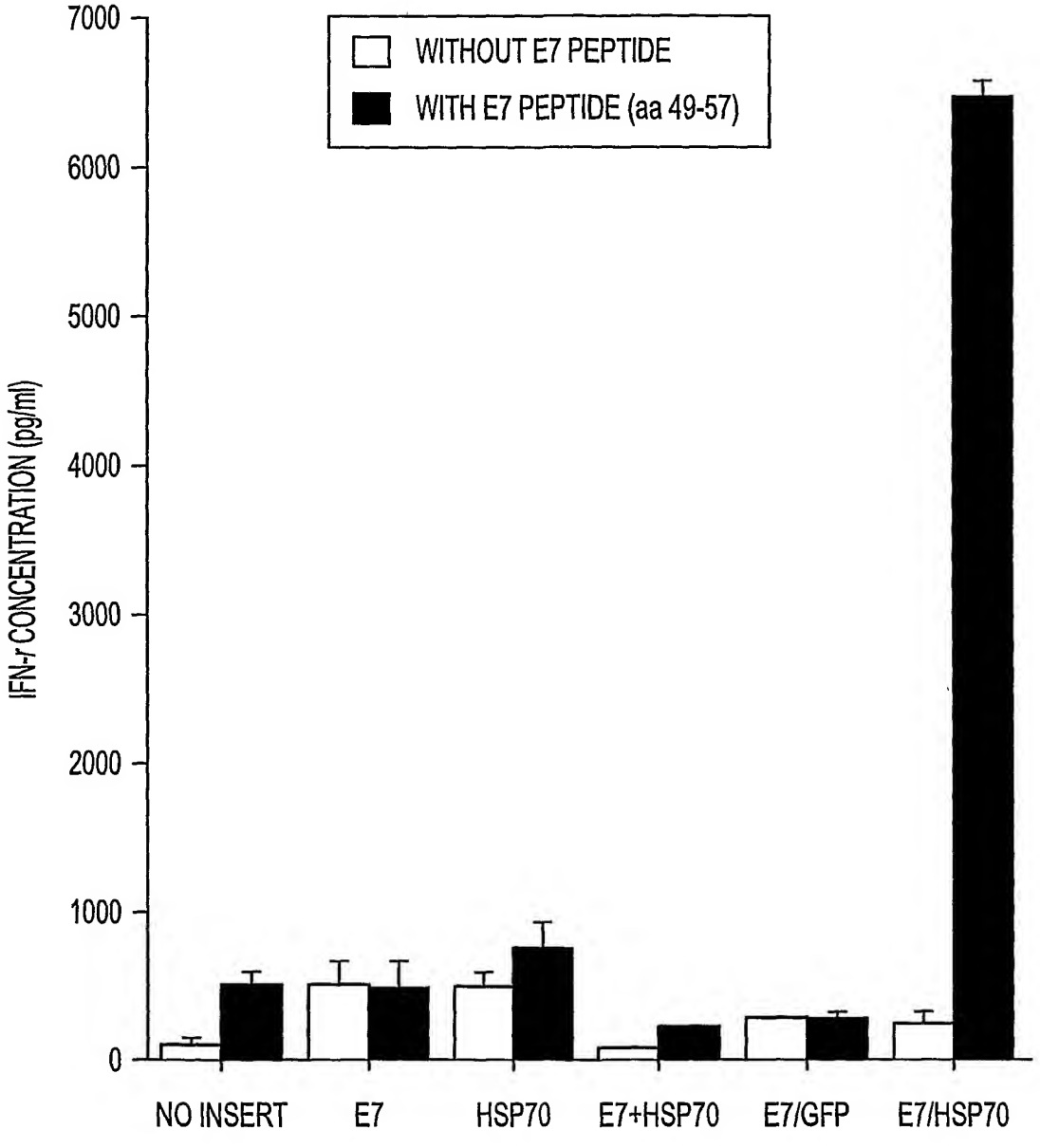

WITHOUT E7 PEPTIDE
WITH E7 PEPTIDE (aa 49-57)
IFN-γ CONCENTRATION (pg/ml)
7000
6000
5000
4000
3000
2000
1000
0
NO INSERT
E7
HSP70
E7+HSP70
E7/GFP
E7/HSP70

FIG. 3

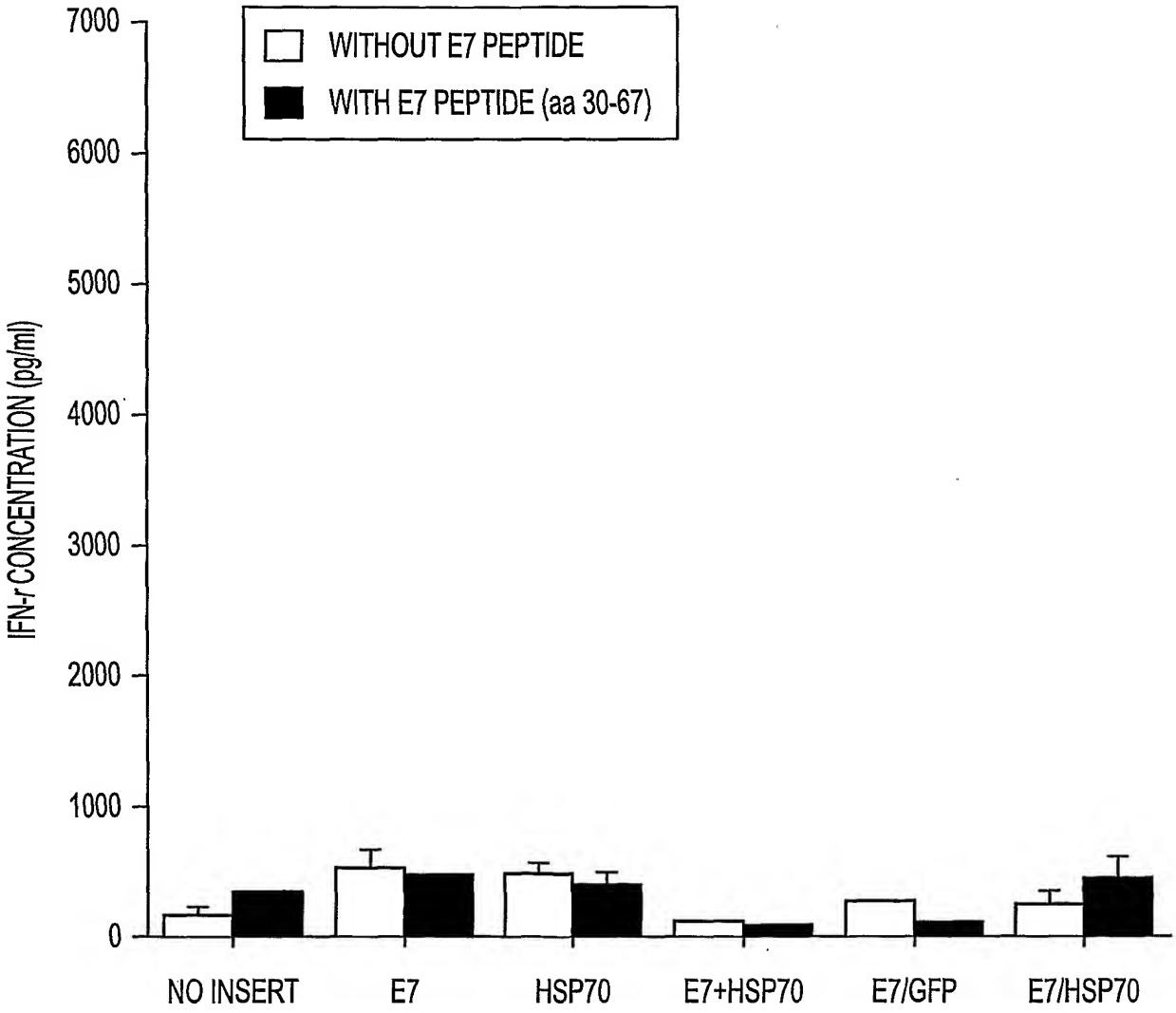

WITHOUT E7 PEPTIDE
WITH E7 PEPTIDE (aa 30-67)
IFN-r CONCENTRATION (pg/ml)
7000
6000
5000
4000
3000
2000
1000
0
NO INSERT
E7
HSP70
E7+HSP70
E7/GFP
E7/HSP70

FIG. 4

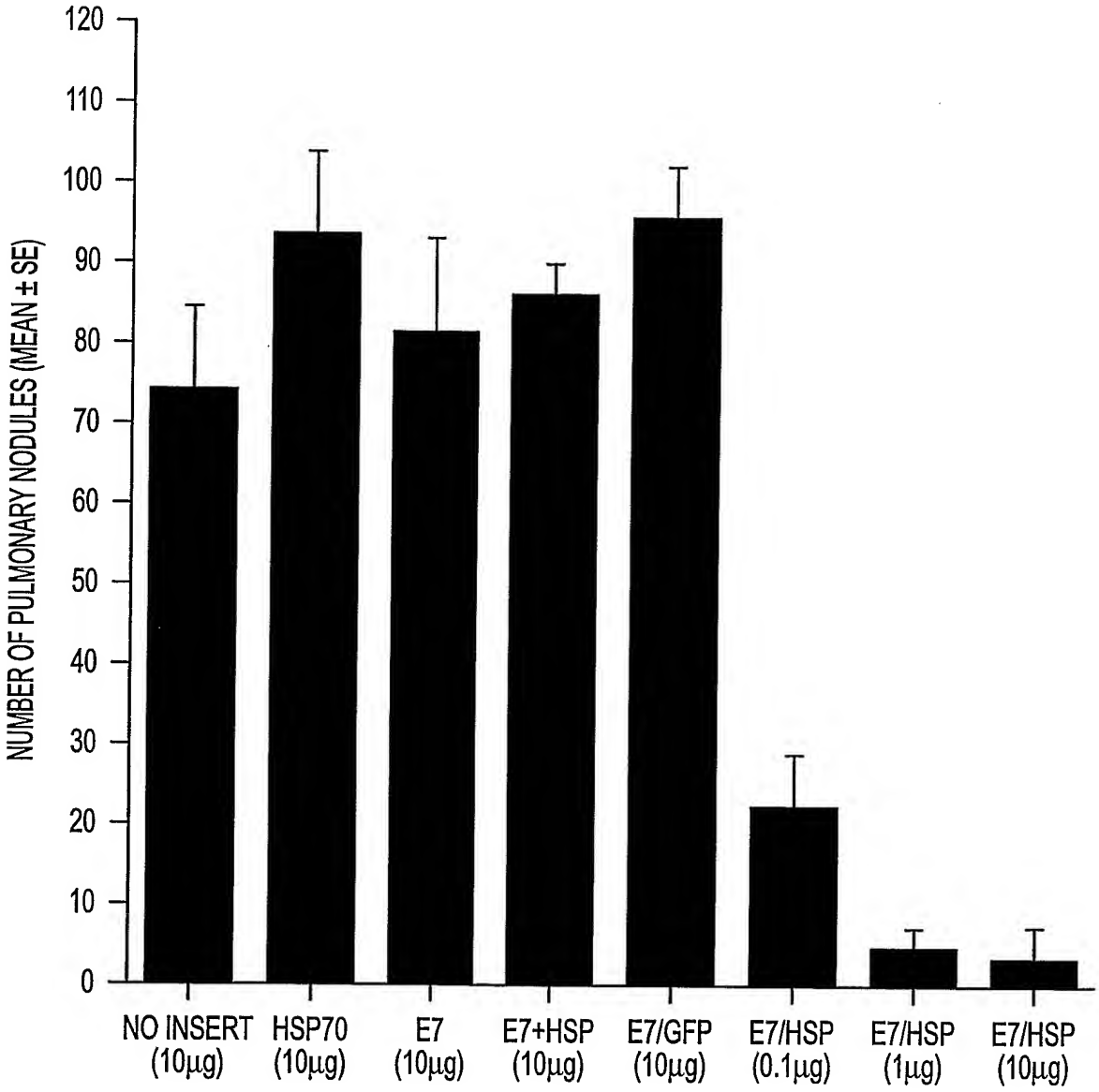

NUMBER OF PULMONARY NODULES (MEAN ± SE)
120
110
100
90
80
70
60
50
40
30
20
10
0
NO INSERT (10μg)
HSP70 (10μg)
E7 (10μg)
E7+HSP (10μg)
E7/GFP (10μg)
E7/HSP (0.1μg)
E7/HSP (1μg)
E7/HSP (10μg)

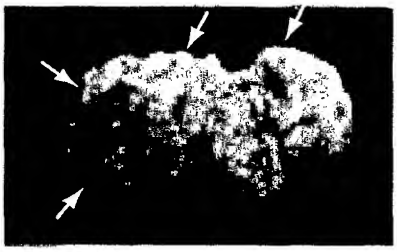
FIG. 5

NO INSERT

FIG. 6A

HSP 70

FIG. 6B

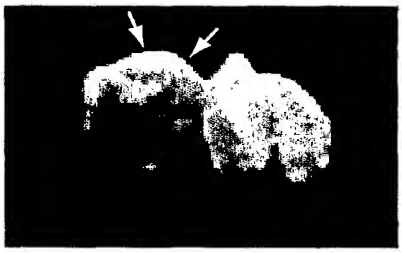


E7

FIG.6C

E7/HSP 70 (0.1μg)

FIG.6D



E7/HSP 70 (1μg)

FIG. 6E

E7/HSP 70 (10μg)

FIG. 6F

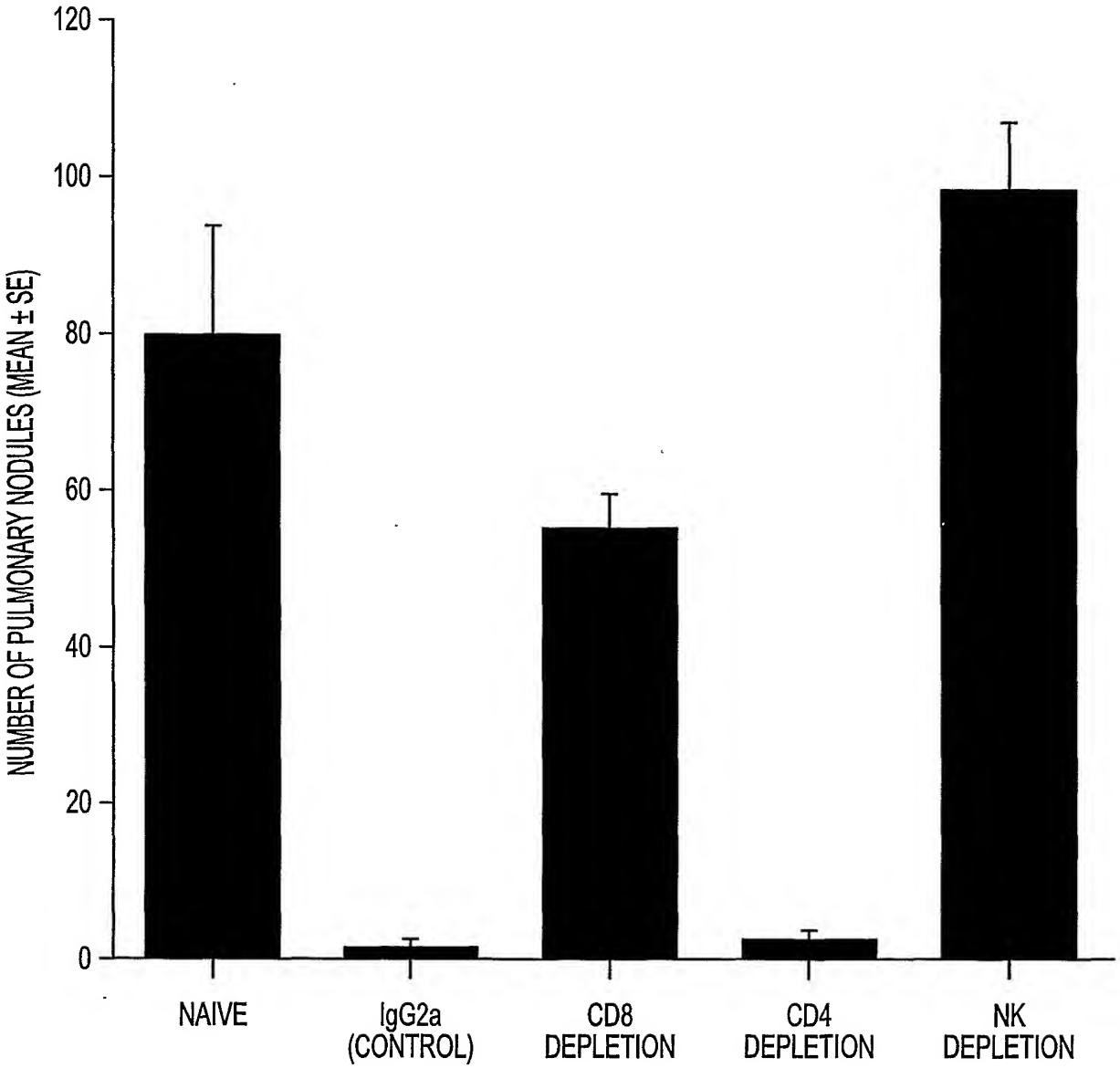

NUMBER OF PULMONARY NODULES (MEAN ± SE)
120
100
80
60
40
20
0
NAIVE
IgG2a (CONTROL)
CD8 DEPLETION
CD4 DEPLETION
NK DEPLETION

FIG. 7

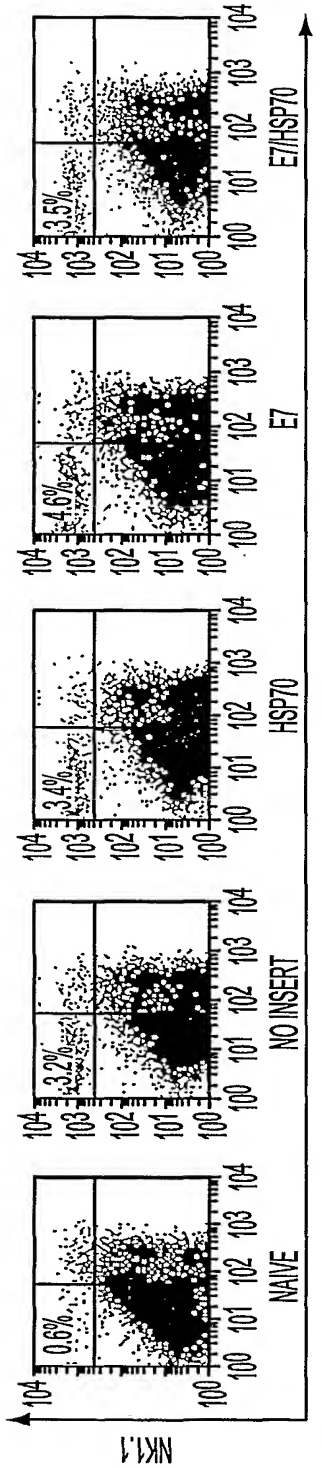

NK1.1
CD3
NAIVE
NO INSERT
HSP70
E7
E7/HSP70
0.6%
3.2%
3.4%
4.6%
3.5%

FIG. 8A

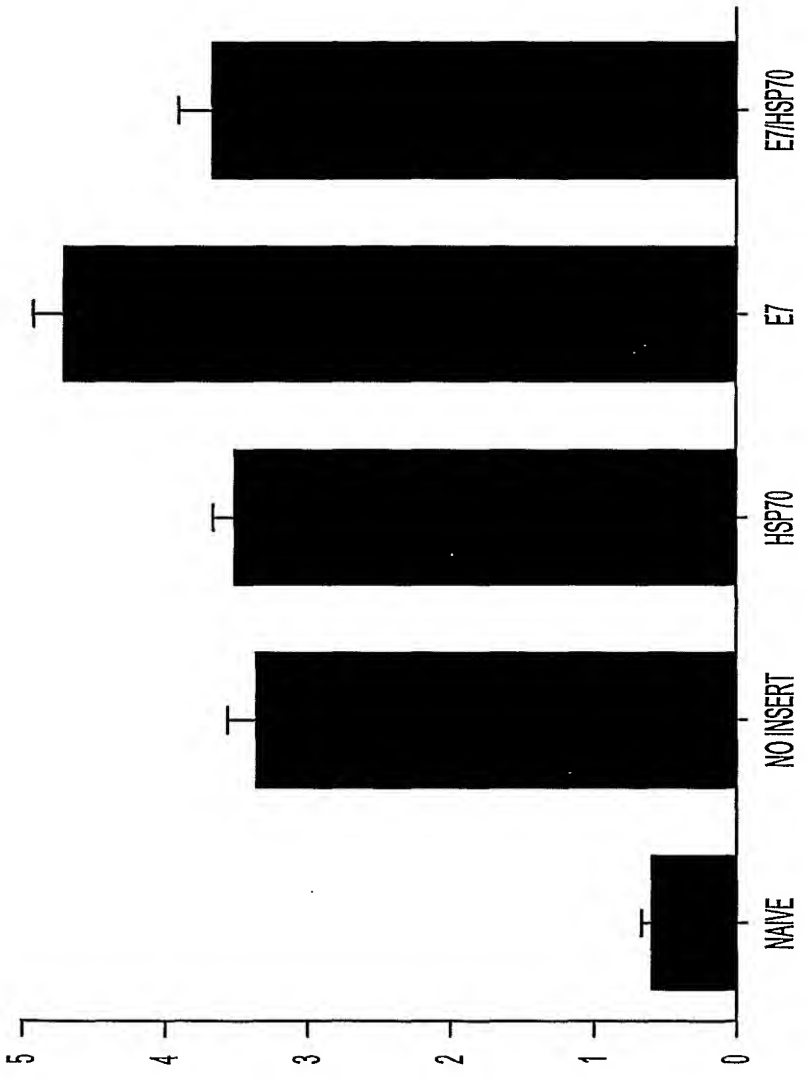

PERCENTAGE OF NK CELLS
0
1
2
3
4
5
NAIVE
NO INSERT
HSP70
E7
E7/HSP70

FIG. 8B

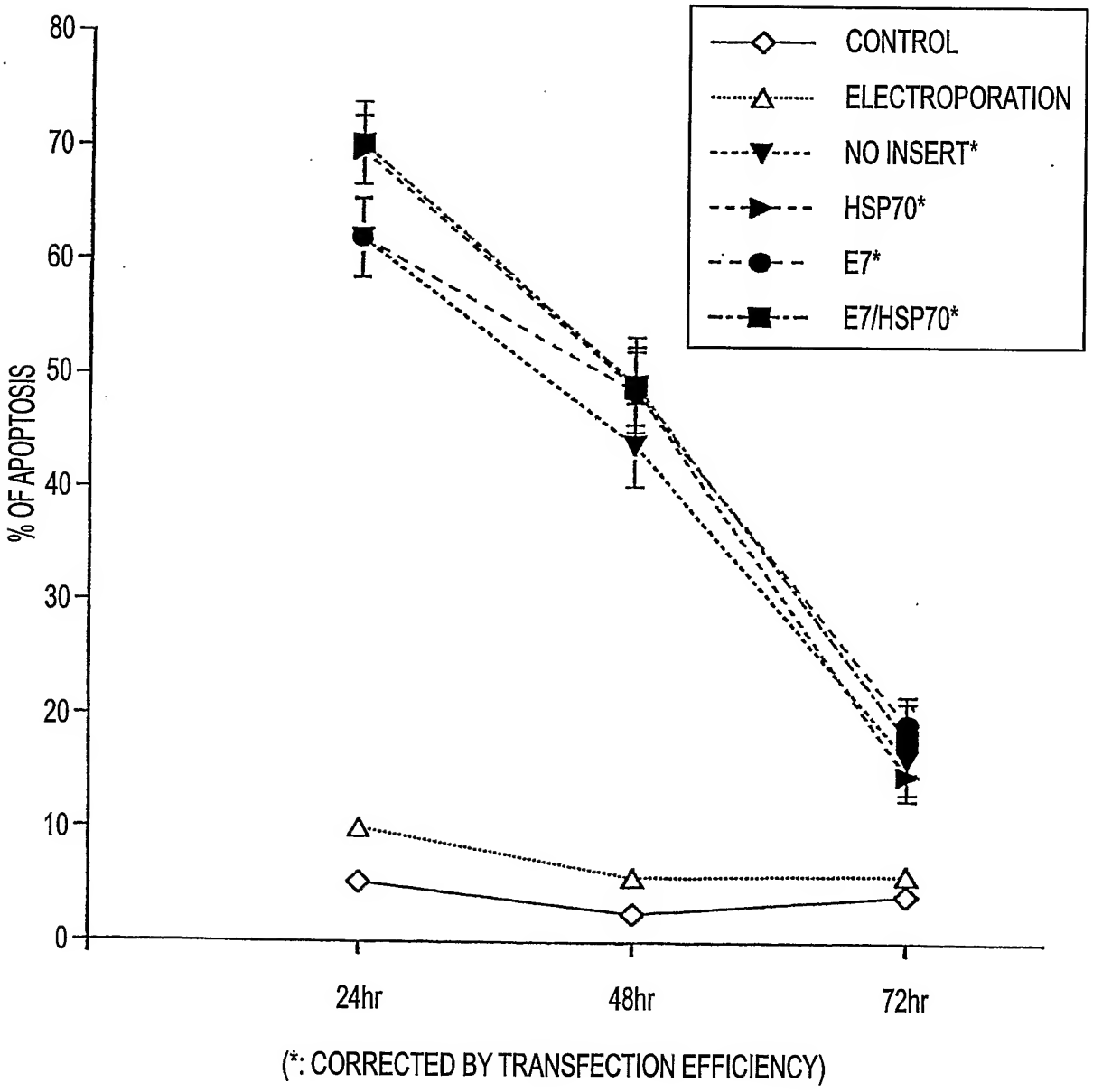

80
70
60
50
40
30
20
10
0
% OF APOPTOSIS
CONTROL
ELECTROPORATION
NO INSERT*
HSP70*
E7*
E7/HSP70*
24hr
48hr
72hr
(*: CORRECTED BY TRANSFECTION EFFICIENCY)

FIG. 9

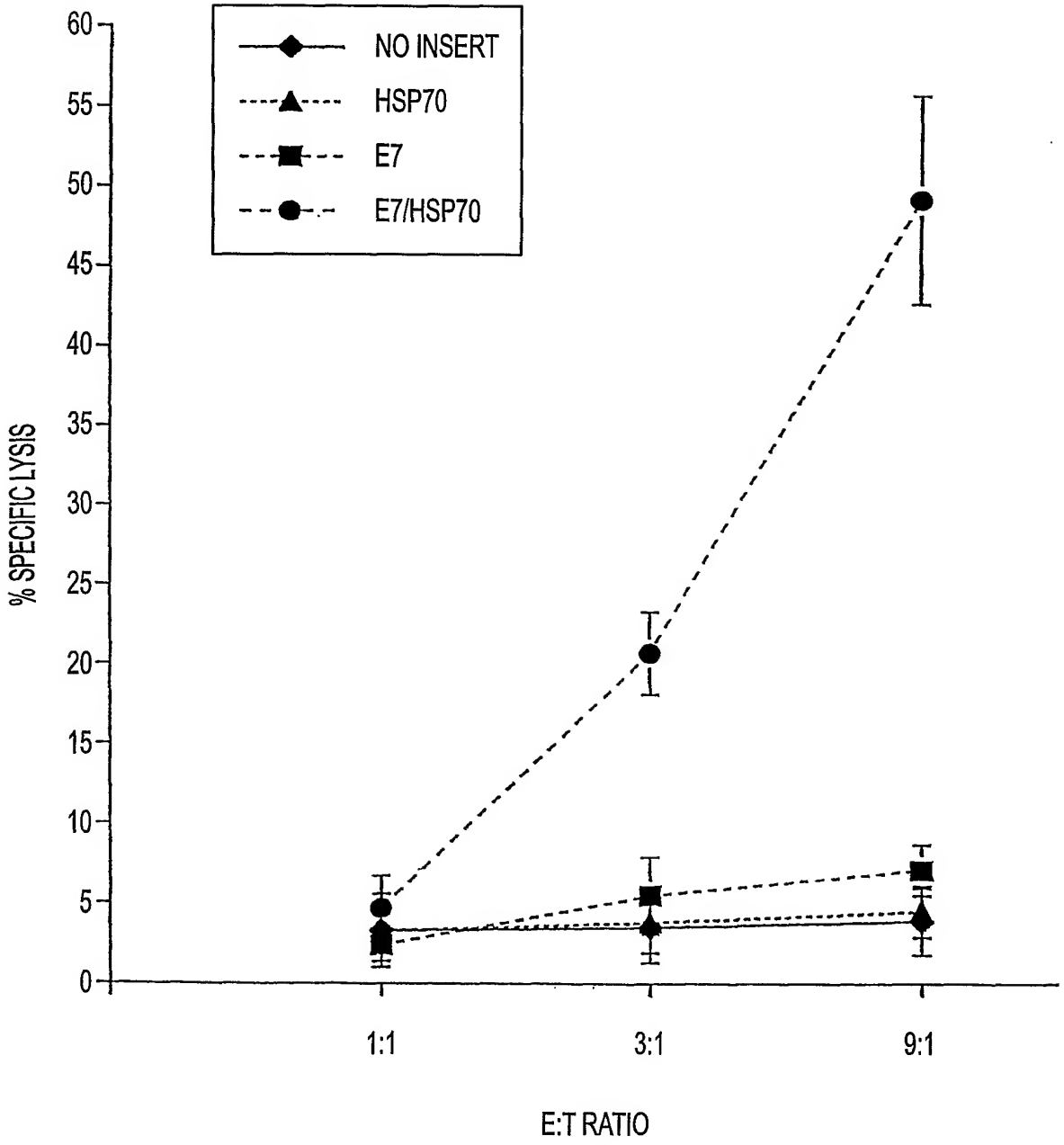

NO INSERT
HSP70
E7
E7/HSP70
% SPECIFIC LYSIS
60
55
50
45
40
35
30
25
20
15
10
5
0
1:1
3:1
9:1
E:T RATIO

FIG. 10

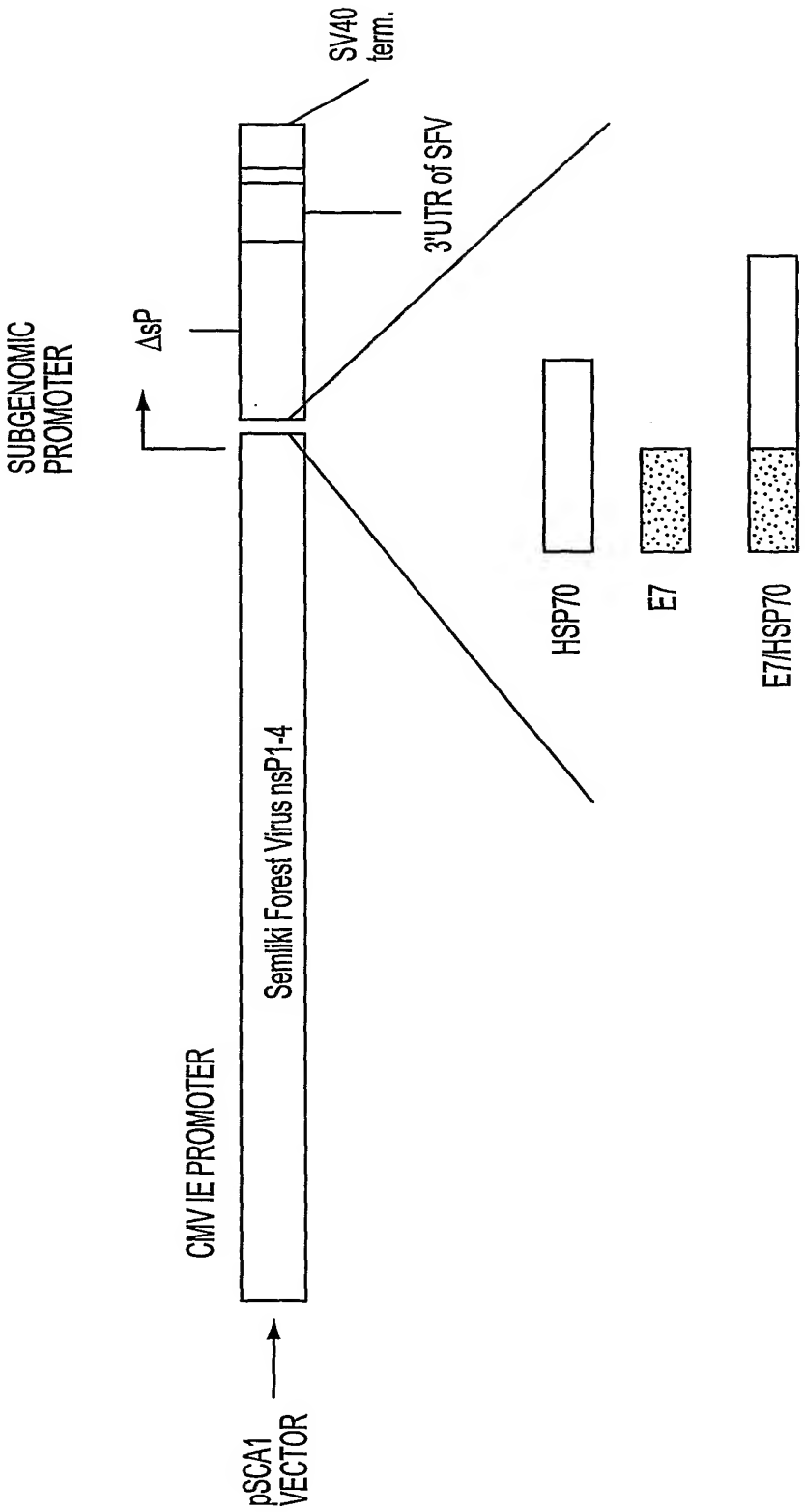

SV40 term.
3'UTR of SFV
SUBGENOMIC PROMOTER
ΔsP
HSP70
E7
E7/HSP70
CMV IE PROMOTER
Semliki Forest Virus nsP1-4
pSCA1 VECTOR

FIG. 11

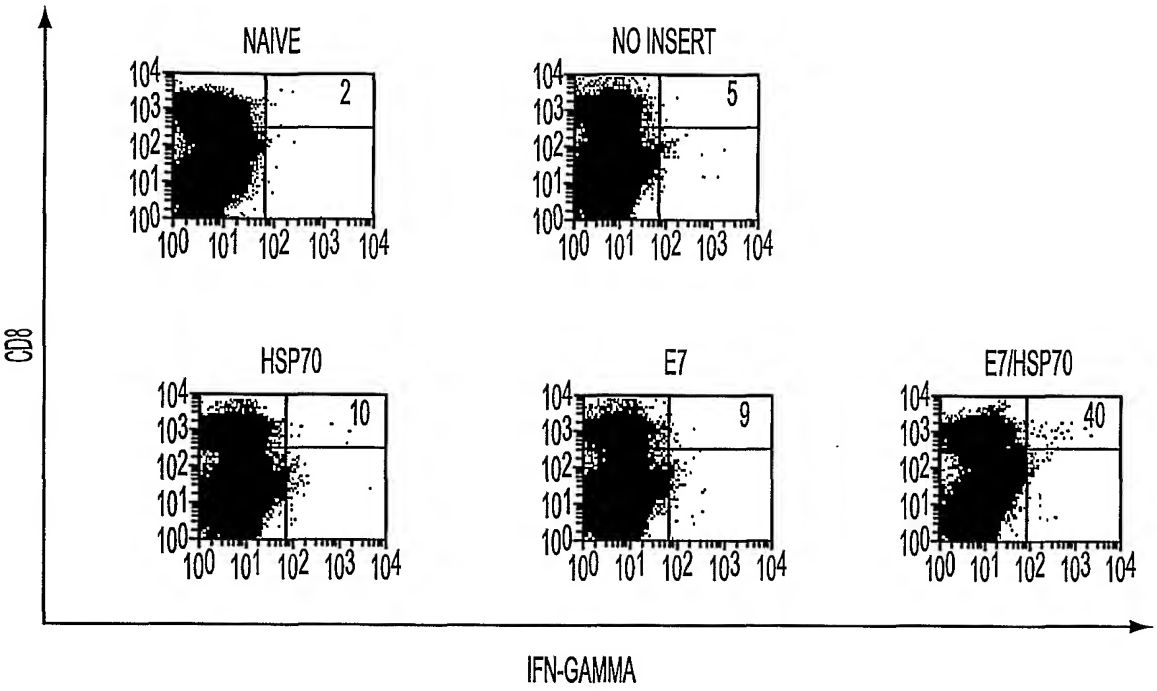


FIG. 12A

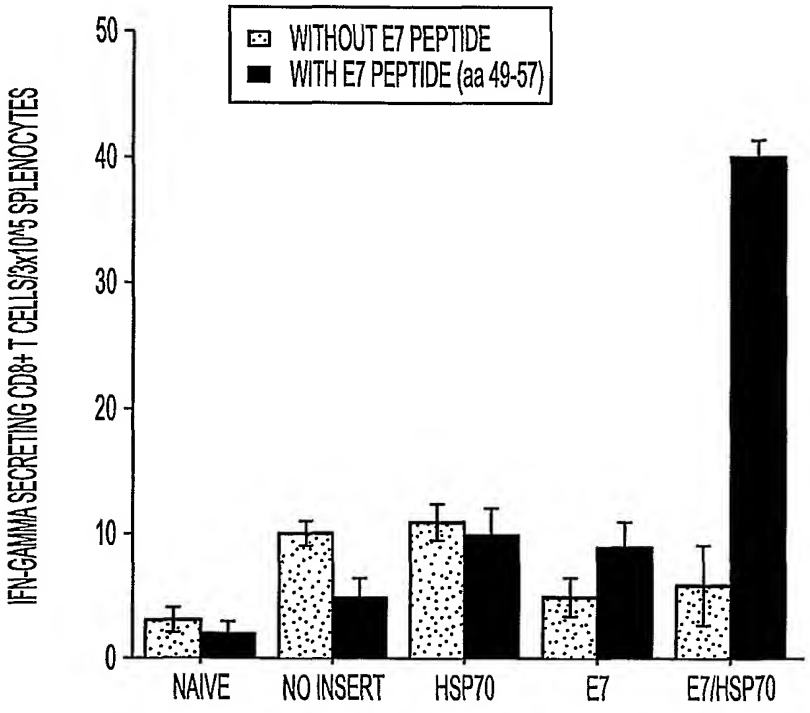


FIG. 12B

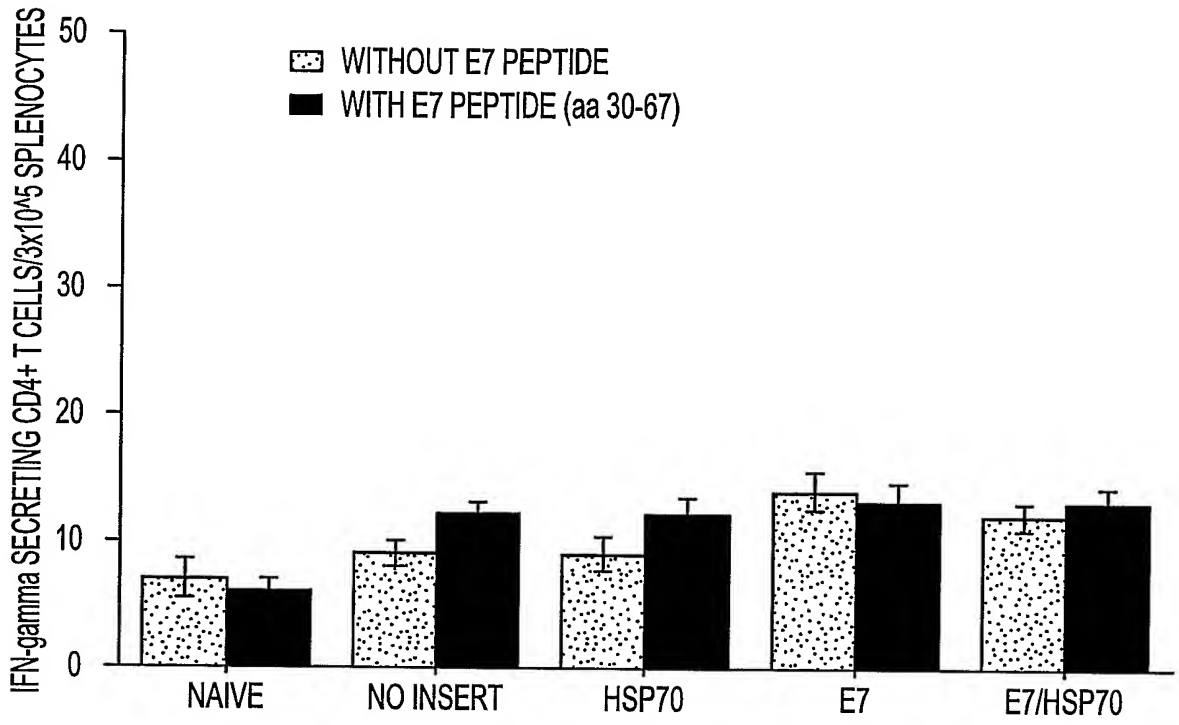


FIG. 13A

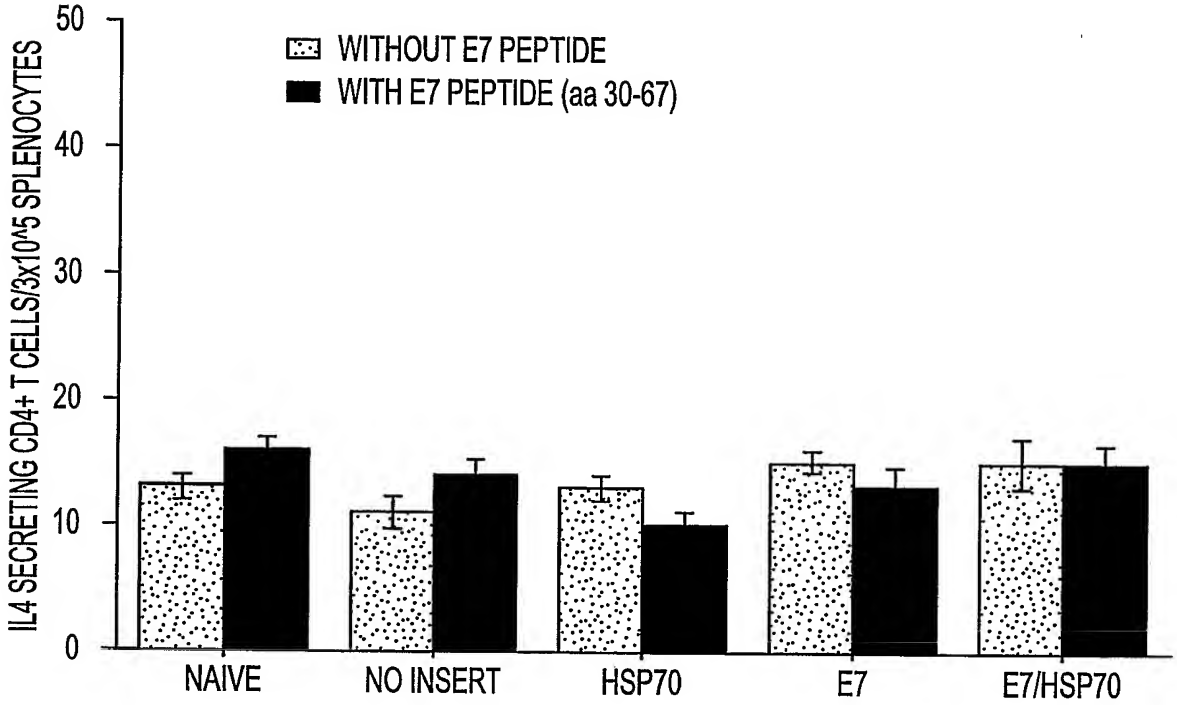


FIG. 13B

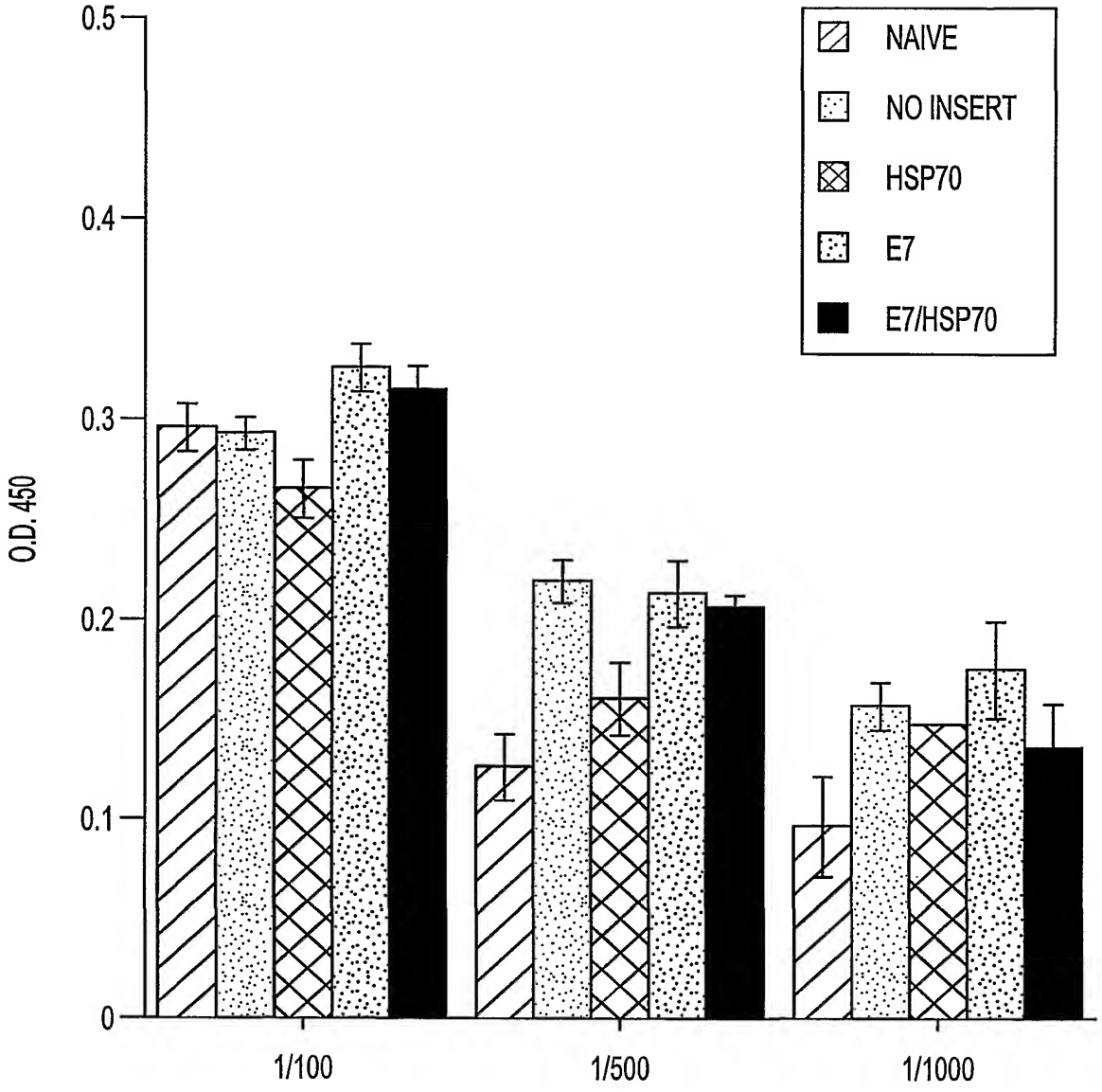

0.5
0.4
0.3
0.2
0.1
0
O.D. 450
1/100
1/500
1/1000
NAIVE
NO INSERT
HSP70
E7
E7/HSP70

FIG. 14

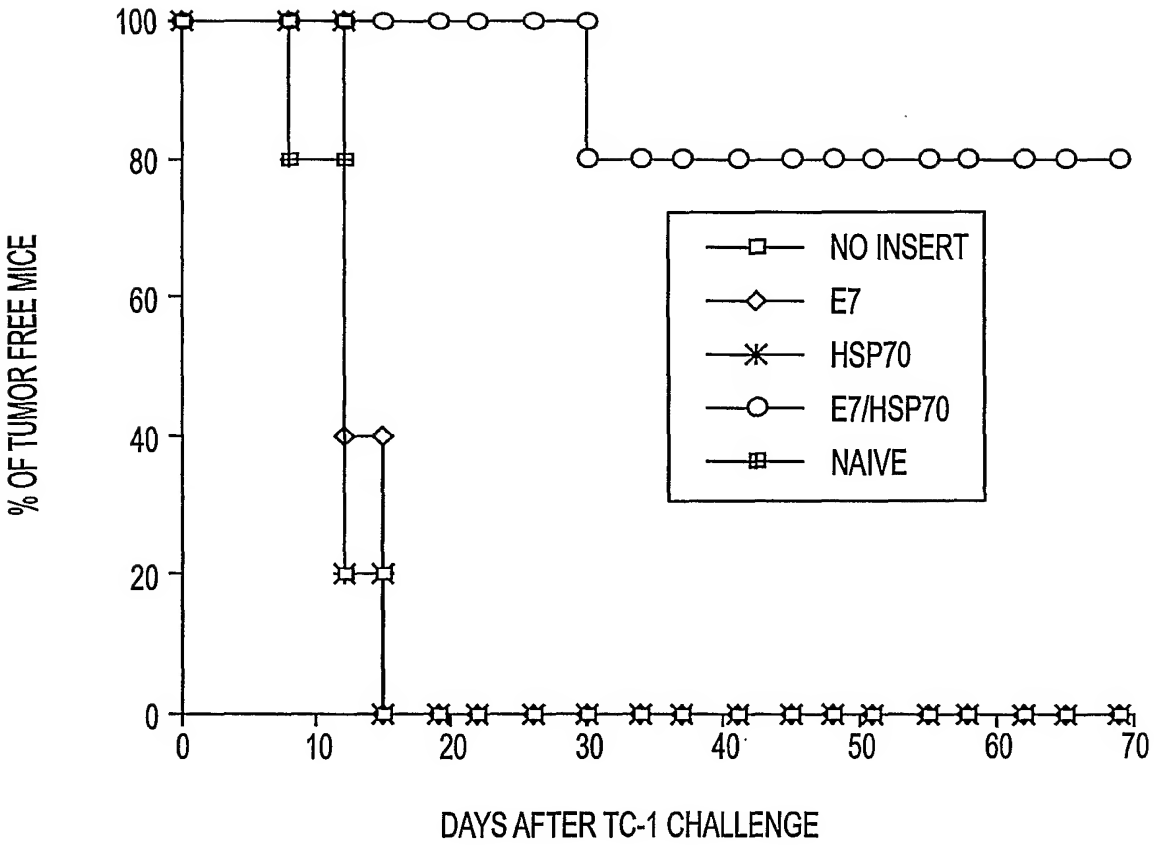

100
80
60
40
20
0
% OF TUMOR FREE MICE
0
10
20
30
40
50
60
70
DAYS AFTER TC-1 CHALLENGE
NO INSERT
E7
HSP70
E7/HSP70
NAIVE

FIG. 15

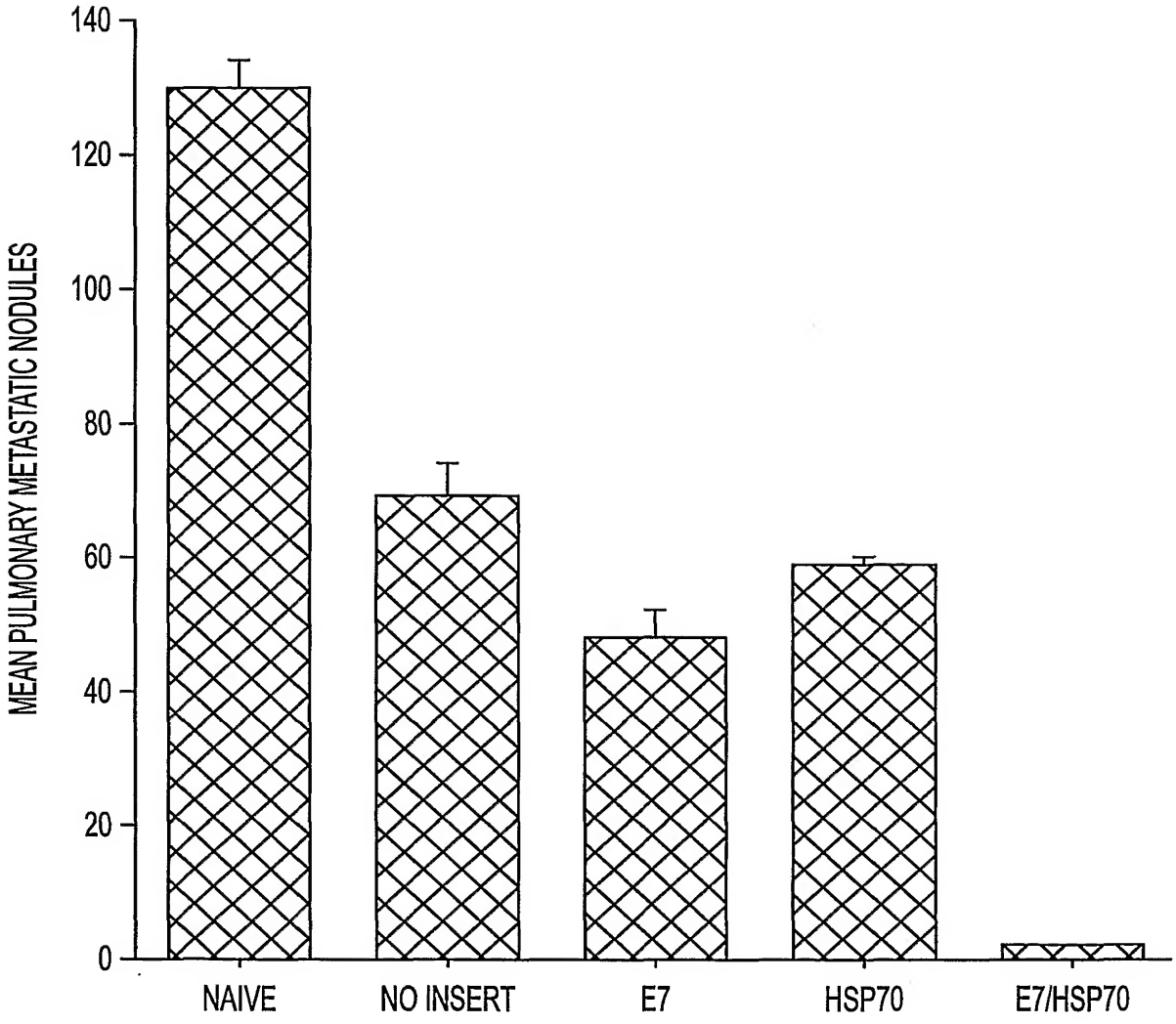

MEAN PULMONARY METASTATIC NODULES
140
120
100
80
60
40
20
0
NAIVE
NO INSERT
E7
HSP70
E7/HSP70

FIG. 16A

FIG. 16B-1

FIG. 16B-2

FIG. 16B-3

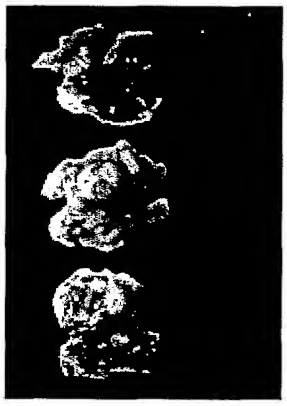


FIG. 16B-4

FIG. 16B-5

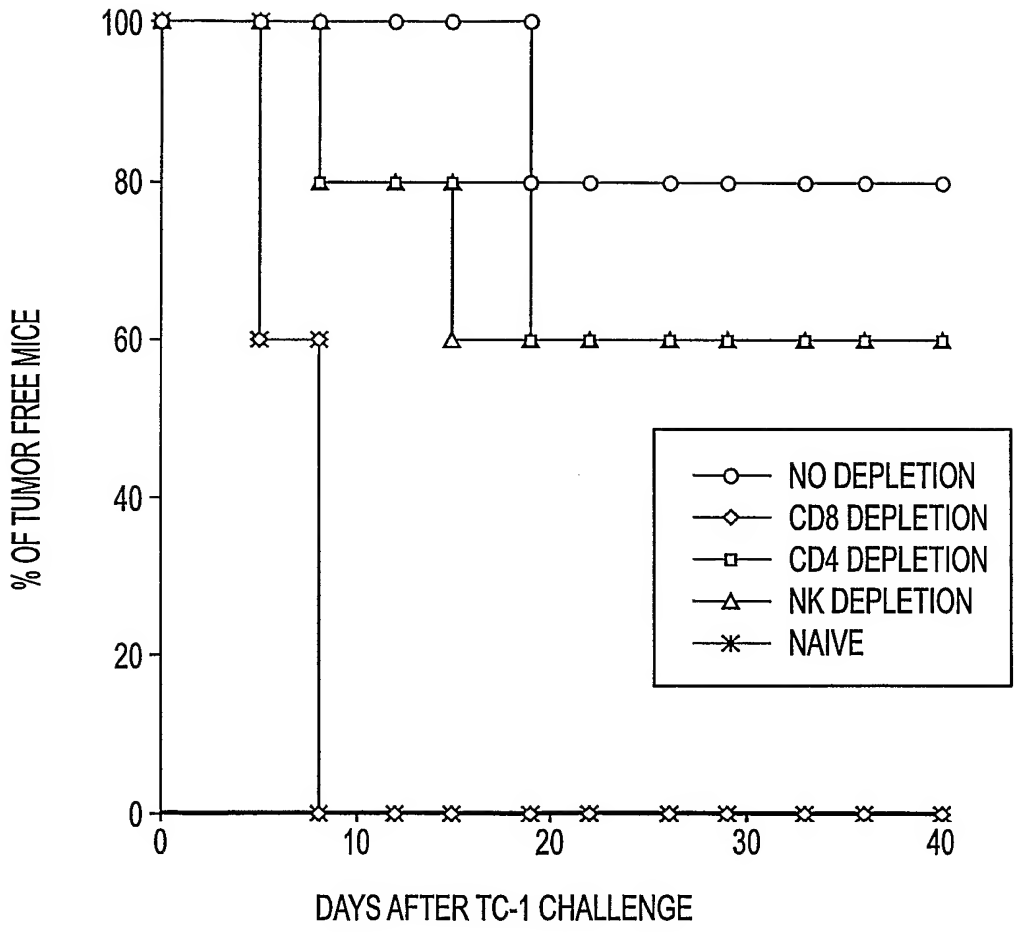

100
80
60
40
20
0
% OF TUMOR FREE MICE
0
10
20
30
40
DAYS AFTER TC-1 CHALLENGE
NO DEPLETION
CD8 DEPLETION
CD4 DEPLETION
NK DEPLETION
NAIVE

FIG. 17

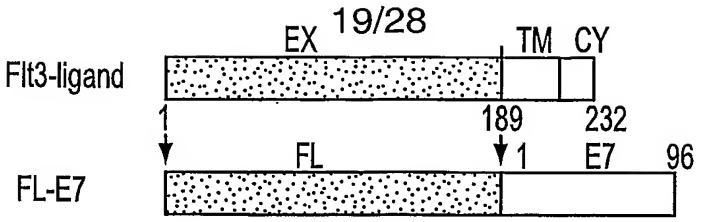


FIG. 18A

1/1 31/11
atg aca gtg ctg gcg cca gcc tgg agc cca aat tcc tcc ctg ttg ctg ctg ttg ctg ctg
Met thr val leu ala pro ala trp ser pro asn ser ser leu leu leu leu leu leu leu
61/21 91/31
ctg agt cct tgc ctg cgg ggg aca cct gac tgt tac ttc agc cac agt ccc atc tcc tcc
leu ser pro cys leu arg gly thr pro asp cys tyr phe ser his ser pro ile ser ser
121/41 151/51
aac ttc aaa gtg aag ttt aga gag ttg act gac cac ctg ctt aaa gat tac cca gtc act
asn phe lys val lys phe arg glu leu thr asp his leu leu lys asp tyr pro val thr
181/61 211/71
gtg gcc gtc aat ctt cag gac gag aag cac tgc aag gcc ttg tgg agc ctc ttc cta gcc
val ala val asn leu gln asp glu lys his cys lys ala leu trp ser leu phe leu ala
241/81 271/91
cag cgc tgg ata gag caa ctg aag act gtg gca ggg tct aag atg caa acg ctt ctg gag
gln arg trp ile glu gln leu lys thr val ala gly ser lys met gln thr leu leu glu
301/101 331/111
gac gtc aac acc gag ata cat ttt gtc acc tca tgt acc ttc cag ccc cta cca gaa tgt
asp val asn thr glu ile his phe val thr ser cys thr phe gln pro leu pro glu cys
361/121 391/131
ctg cga ttc gtc cag acc aac atc tcc cac ctc ctg aag gac acc tgc aca cag ctg ctt
leu arg phe val gln thr asn ile ser his leu leu lys asp thr cys thr gln leu leu
421/141 451/151
gct ctg aag ccc tgt atc ggg aag gcc tgc cag aat ttc tct cgg tgc ctg gag gtg cag
ala leu lys pro cys ile gly lys ala cys gln asn phe ser arg cys leu glu val gln
481/161 511/171
tgc cag ccg gac tcc tcc acc ctg ctg ccc cca agg agt ccc ata gcc cta gaa gcc acg
cys gln pro asp ser ser thr leu leu pro pro arg ser pro ile ala leu glu ala thr
541/181 571/191
gag ctc cca gag cct cgg ccc agg cag gga tcc atg cat gga gat aca cct aca ttg cat
glu leu pro glu pro arg pro arg gln *gly* ser met his gly asp thr pro thr leu his
601/201 631/211
gaa tat atg tta gat ttg caa cca gag aca act gat ctc tac tgt tat gag caa tta aat
glu tyr met leu asp leu gln pro glu thr thr asp leu tyr cys tyr glu gln leu asn
661/221 691/231
gac agc tca gag gag gag gat gaa ata gat ggt cca gct gga caa gca gaa ccg gac aga
asp ser ser glu glu glu asp glu ile asp gly pro ala gly gln ala glu pro asp arg
721/241 751/251
gcc cat tac aat att gta acc ttt tgt tgc aag tgt gac tct acg ctt cgg ttg tgc gta
ala his tyr asn ile val thr phe cys cys lys cys asp ser thr leu arg leu cys val
781/261 811/271
caa agc aca cac gta gac att cgt act ttg gaa gac ctg tta atg ggc aca cta gga att
gln ser thr his val asp ile arg thr leu glu asp leu leu met gly thr leu gly ile
841/281 871/291
gtg tgc ccc atc tgt tct cag gat aag ctt aag ttt aaa ccg ctg atc agc ctc gac tgt
val cys pro ile cys ser gln asp lys leu lys phe lys pro leu ile ser leu asp cys
901/301
gcc ttc tag
ala phe AMB

FIG. 18B

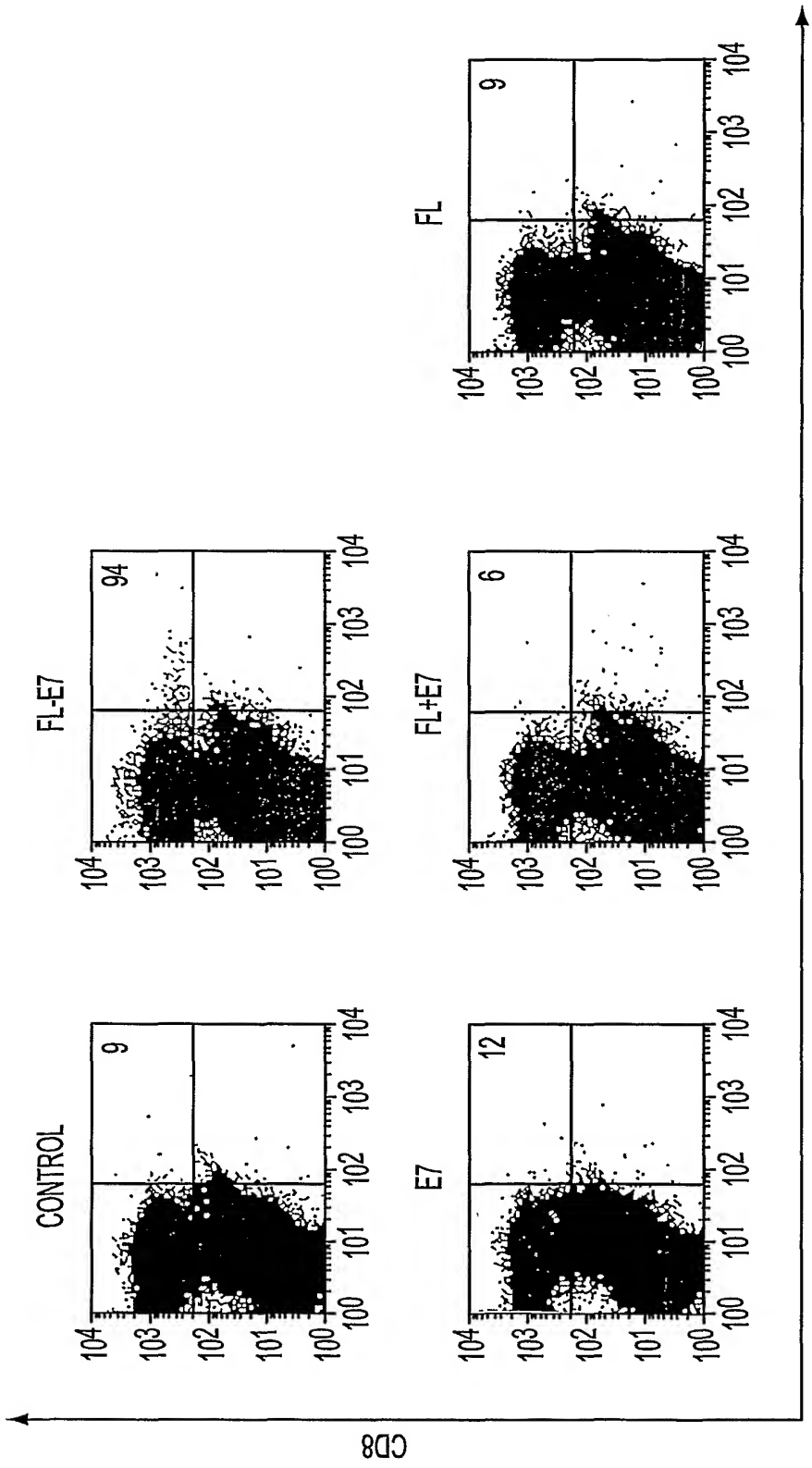


FIG. 19A

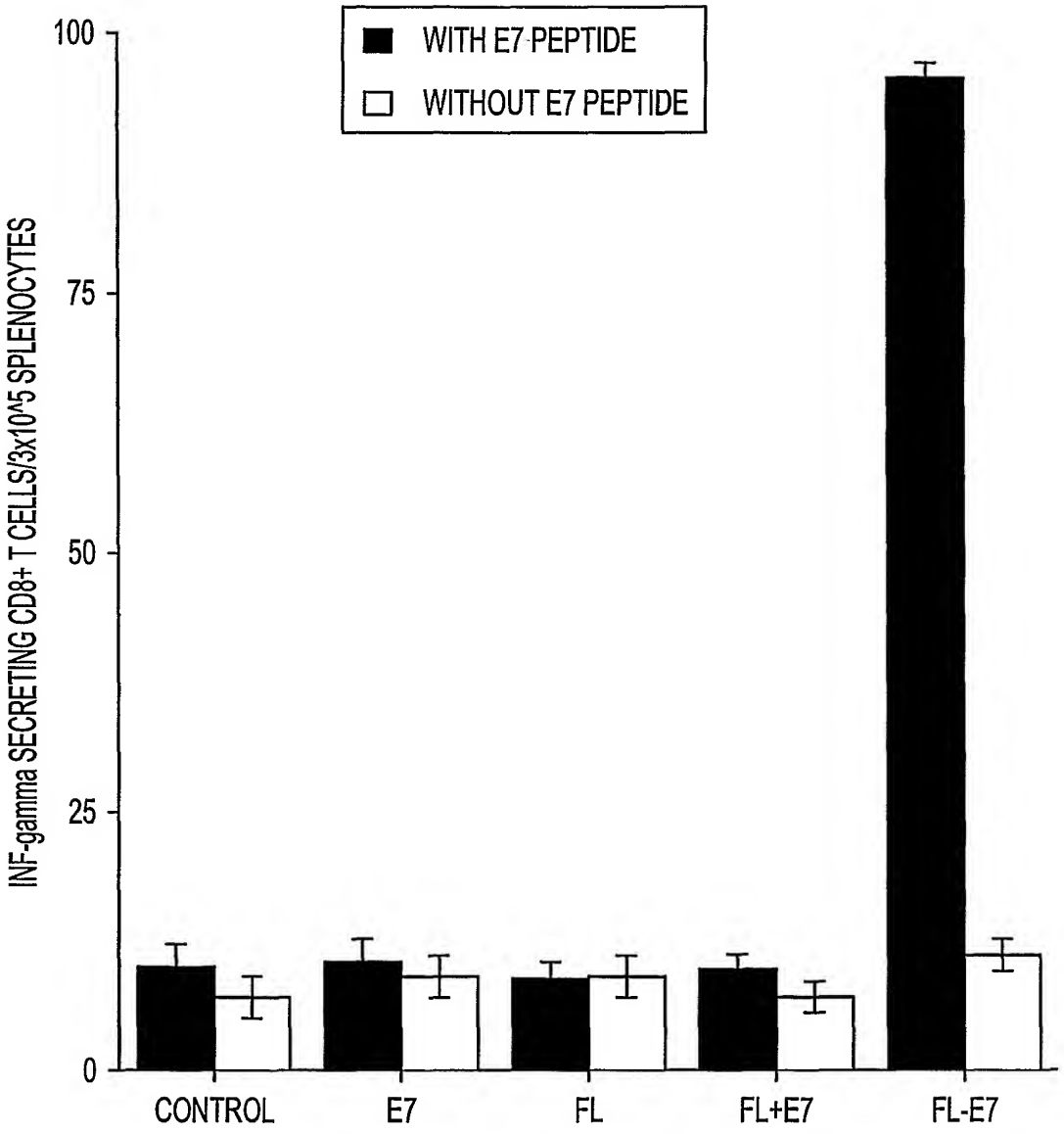

WITH E7 PEPTIDE
WITHOUT E7 PEPTIDE
INF-gamma SECRETING CD8+ T CELLS/3x10^5 SPLENOCYTES
100
75
50
25
0
CONTROL
E7
FL
FL+E7
FL-E7

FIG. 19B

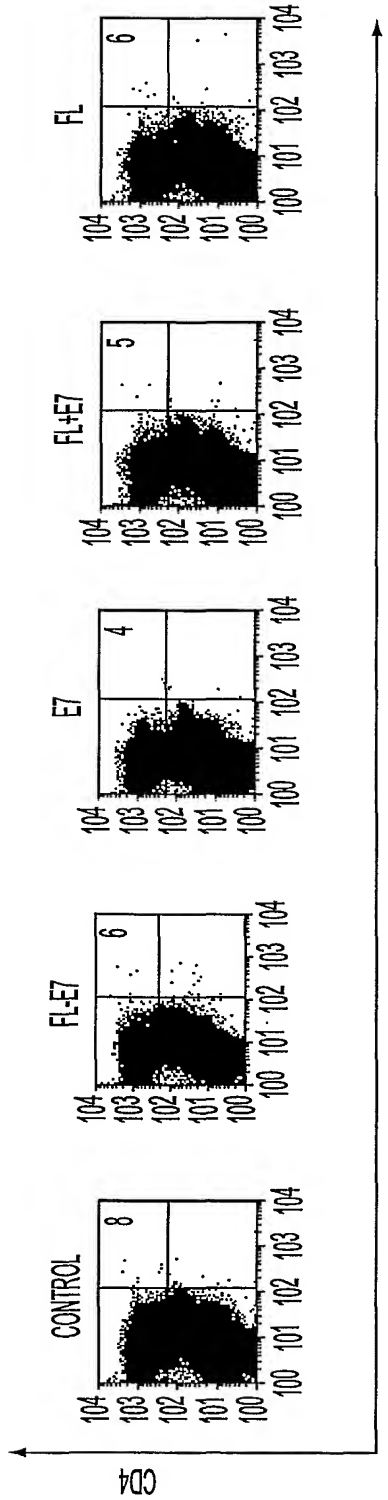


FIG. 20A

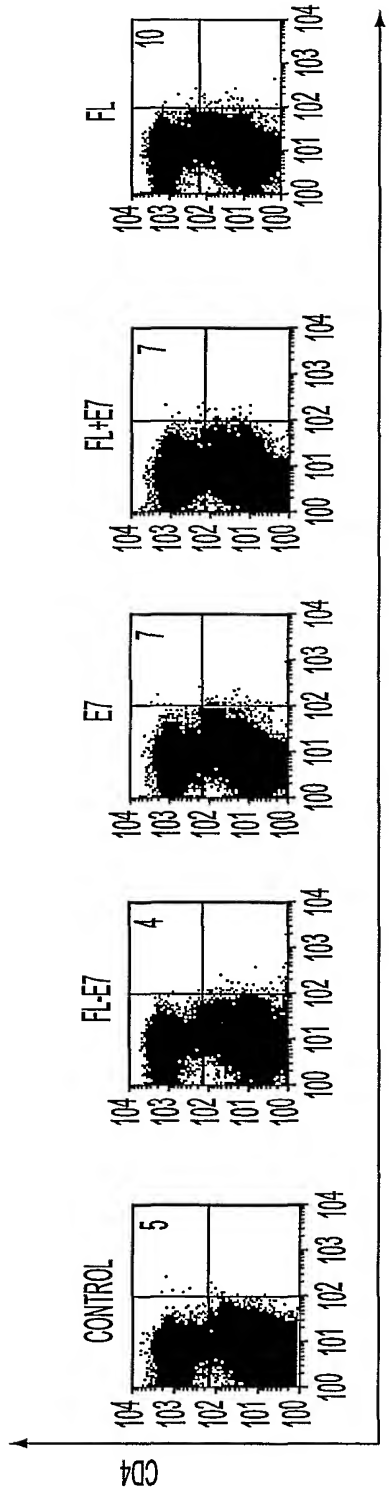


FIG. 20B

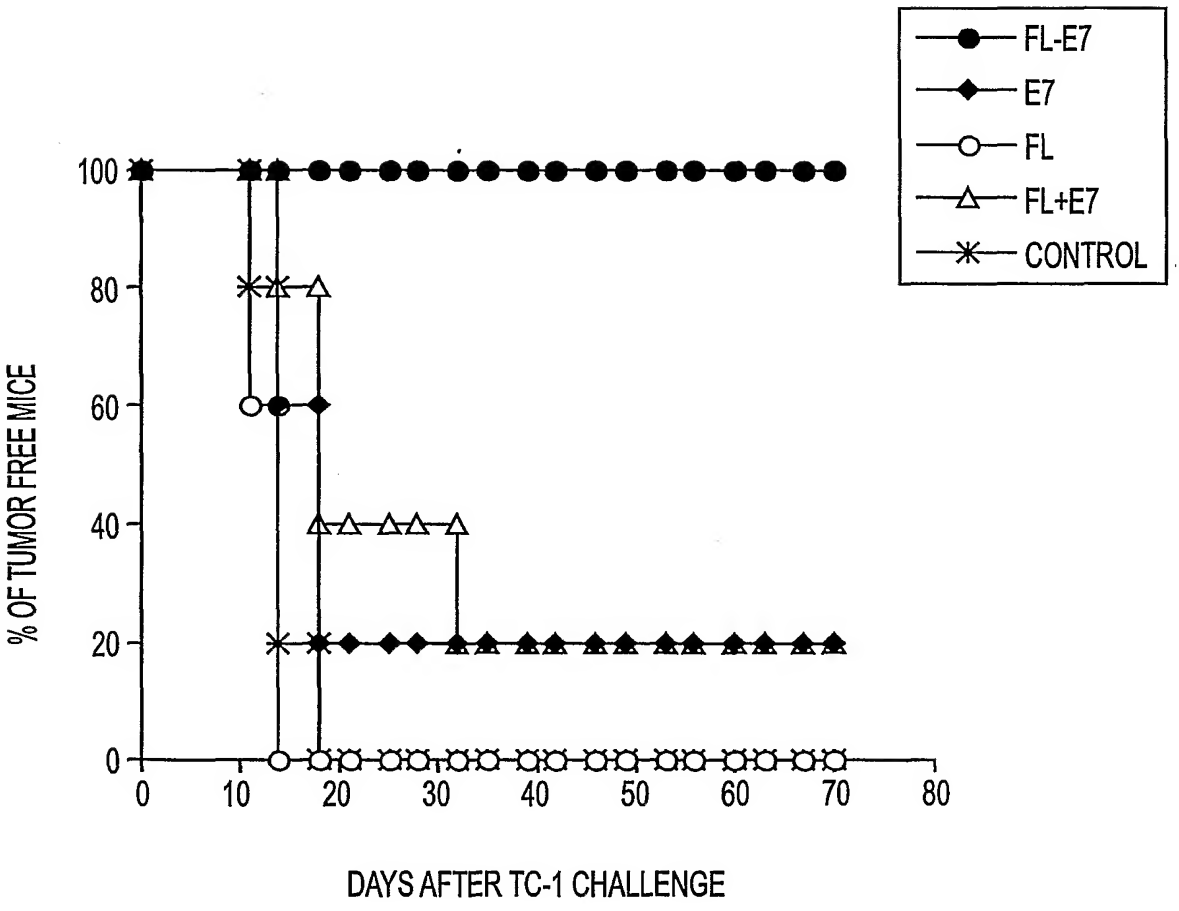

FL-E7
E7
FL
FL+E7
CONTROL
100
80
60
40
20
0
% OF TUMOR FREE MICE
0
10
20
30
40
50
60
70
80
DAYS AFTER TC-1 CHALLENGE

FIG. 21

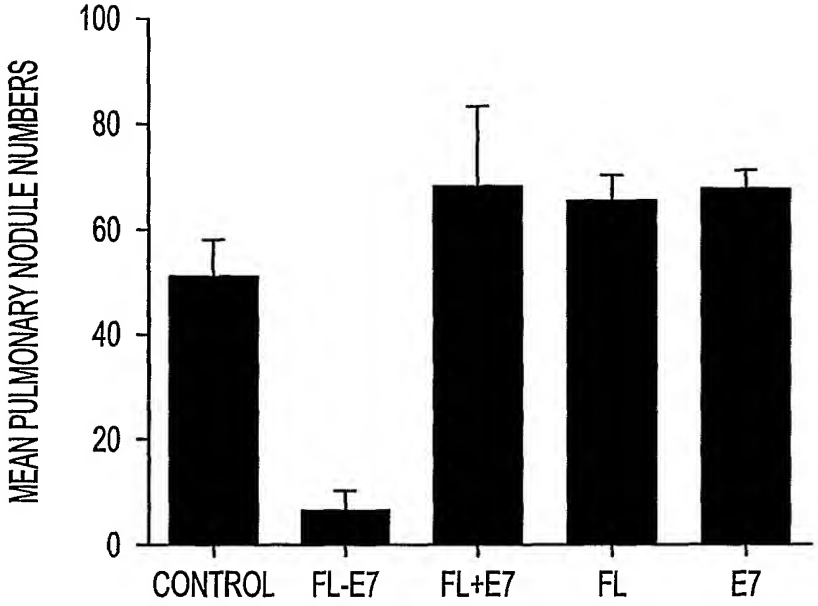


FIG. 22A

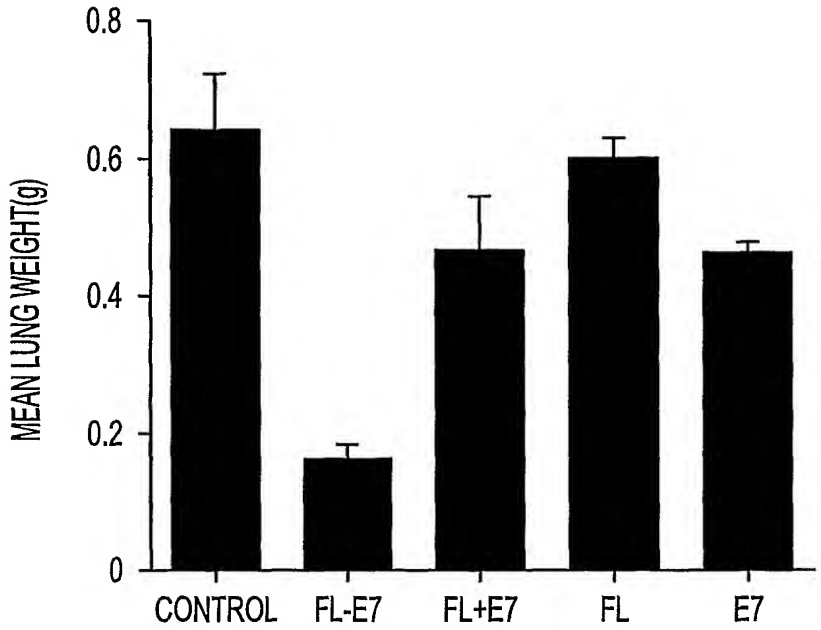


FIG. 22B

FIG. 23A

FIG. 23B

FIG. 23C

FIG. 23D

FIG. 23E

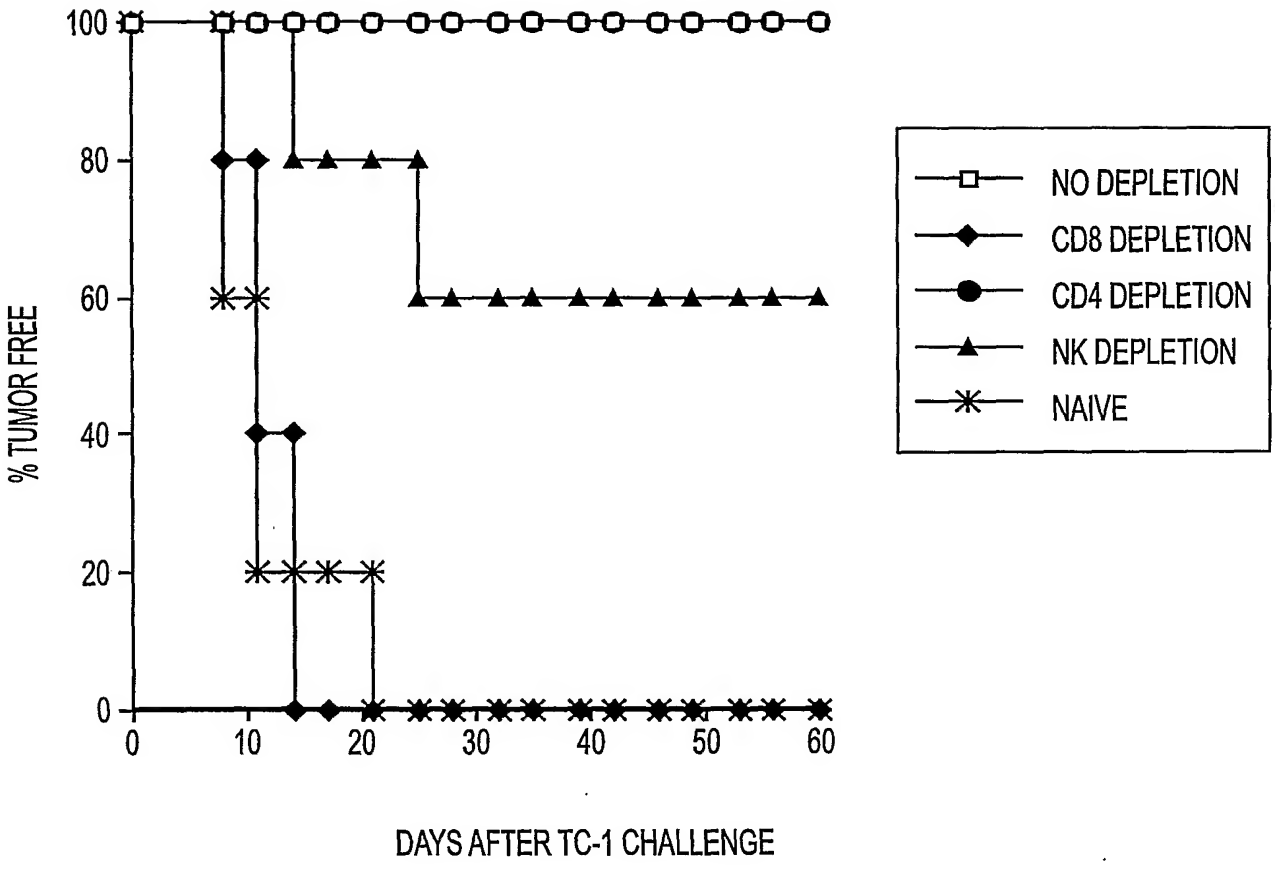

100
80
60
40
20
0
% TUMOR FREE
0
10
20
30
40
50
60
DAYS AFTER TC-1 CHALLENGE
NO DEPLETION
CD8 DEPLETION
CD4 DEPLETION
NK DEPLETION
NAIVE

FIG. 24

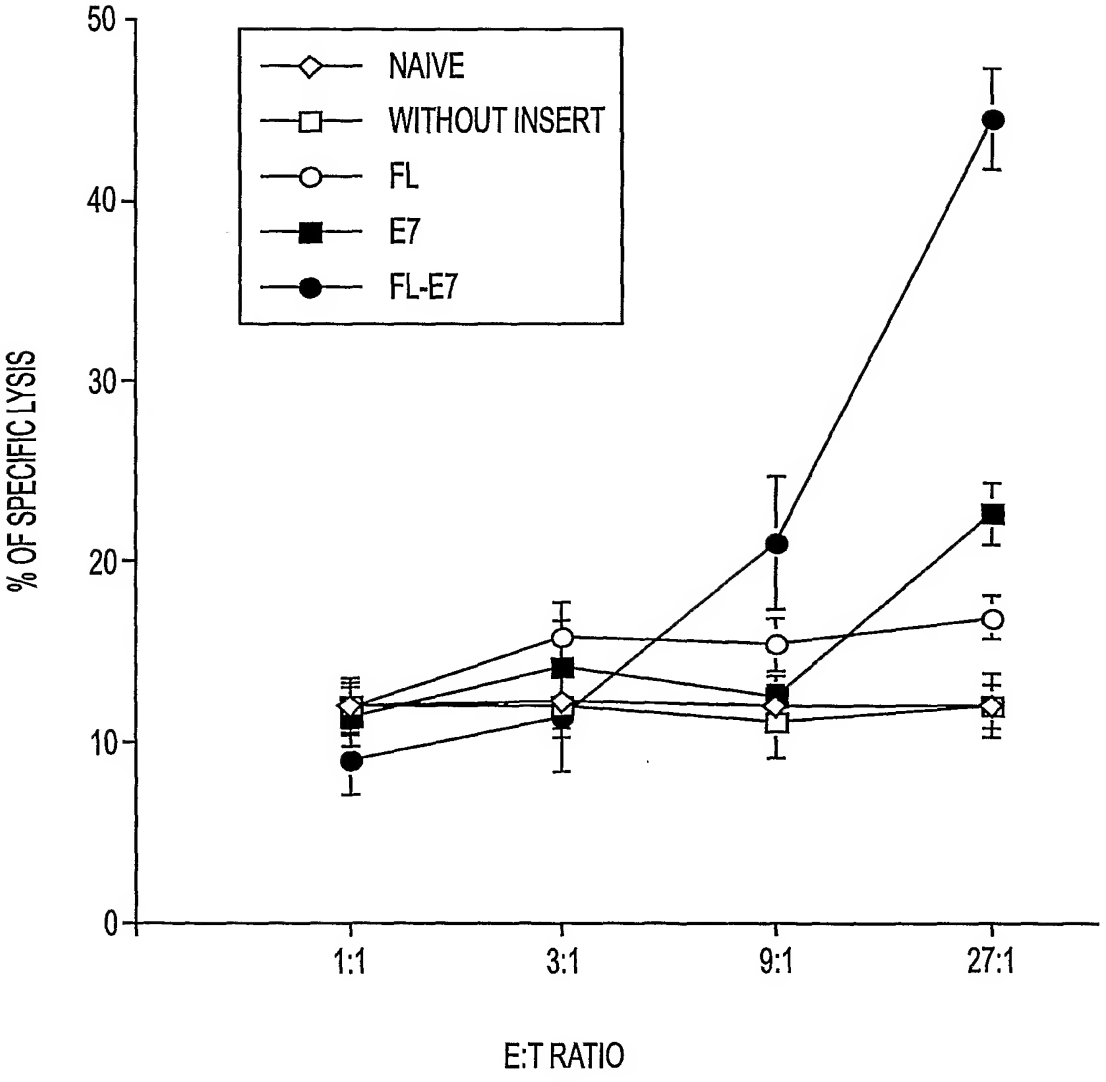

NAIVE
WITHOUT INSERT
FL
E7
FL-E7
% OF SPECIFIC LYSIS
50
40
30
20
10
0
1:1
3:1
9:1
27:1
E:T RATIO

FIG. 25

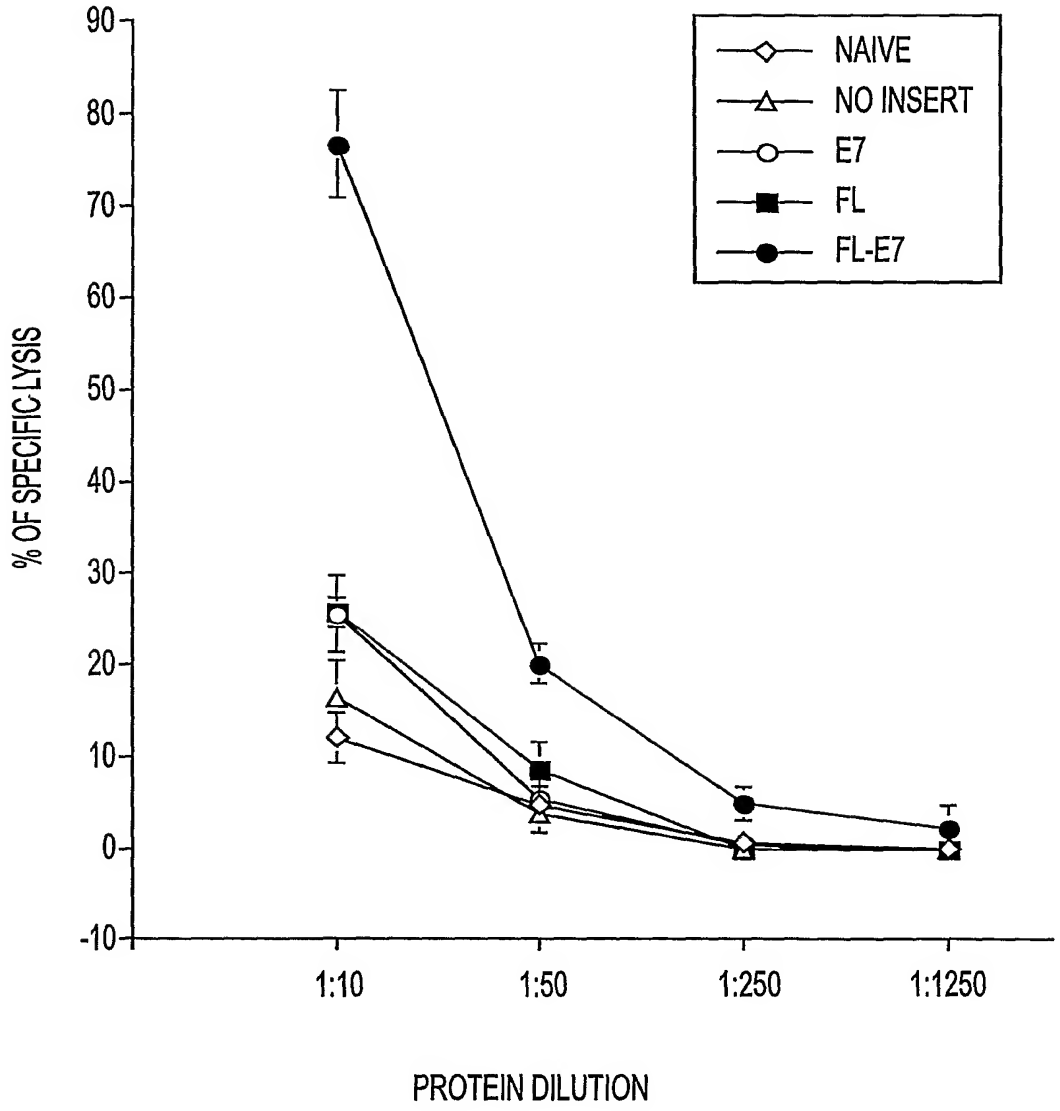

90
80
70
60
50
40
30
20
10
0
-10
% OF SPECIFIC LYSIS
NAIVE
NO INSERT
E7
FL
FL-E7
1:10
1:50
1:250
1:1250
PROTEIN DILUTION

FIG. 26

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/02598

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 39/00; C07H 21/04; C12N 15/00, 15/63, 15/85

US CL : 424/192.1; 435/69.7, 320.1, 325; 536/23.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/192.1; 435/69.7, 320.1, 325; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SUZUE, K. et al. Heat shock fusion proteins as vehicles for antigen delivery into the major histocompatibility complex class I presentation pathway. Proc. Natl. Acad. Sci., USA. November 1997, Vol. 94, pages 1316-13151, entire document.	1-9, 48-50, 56, 60-63 and 75-76
Y	CHU, N. R. et al. Immunotherapy of a human papillomavirus (HPV) type 16 E7-expressing tumour by administration of a fusion protein comprising Mycobacterium bovis bacille Calmette-Guerin (BCG) hsp65 and HPV16 E7. Clin. Exp. Immunol. 2000, Vol. 121, pages 216-225, entire document.	1-9, 48-50, 56, 60-63, 75-76
Y	MORE, S. et al. Activation of cytotoxic T cells in vitro by recombinant gp96 fusion proteins irrespective of the 'fused' antigenic peptide sequence. Immunology Letters. 1991, Vol. 69, pages 275-282, entire document.	1-9, 48-50, 56, 60-63, 75-76
A	CHENG et al. Enhancement of Sindbis Virus Self-Replicating RNA Vaccine Potency by Linkage of Mycobacterium tuberculosis Heat Shock Protein 70 Gene to an Antigen Gene. J. Immunology. 2001, Vol. 166, pages 6218-6226.	1-9, 48-50, 56, 60-63, 75-76

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

06 September 2002 (06.09.2002)

Date of mailing of the international search report

20 SEP 2002

Name and mailing address of the ISA/US

Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703)305-3230

Authorized officer

Amy DeCloux

Telephone No. 703 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/02598

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	HUNG, C.-F. et al. Enhancement of DNA Vaccine Potency by Linkage of Antigen Gene to a Gene Encoding the Extracellular Domain of Fms-like Tyrosine Kinase 3-Ligand. Cancer Research. February 2001, Vol. 61, pages 1080-1088.	1-9, 48-50, 56, 60-63, 75-76

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/02598

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claim Nos.: 10-47, 51-55, 57-59, 67-74 and 77
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/02598

Continuation of Item 4 of the first sheet:
Title is longer than 17 words, PCT Rule 4,3. Suggested new title follows:

"Nucleic Acid derived vaccine that encodes an antigen linked to a polypeptide that promotes antigen presentation".

Continuation of B. FIELDS SEARCHED Item 3:
STN/CAS:Medline, CAPLUS, Embase, Biosis, WEST